

PSEUDOMONAS '97

Madrid, Spain 1-8 September • 1997

I International Congress on *Pseudomonas*:
**Molecular Biology and
Biotechnology**

Abstracts Book

Pseudomonas 97, Madrid 4-8 Sept. 1997

Lecture Hall of the
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Serrano 117, Madrid 28006

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SCIENTIFIC PROGRAM & ABSTRACTS

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Este Congreso ha sido declarado de *Interés Sanitario* por el Ministerio de Sanidad y Consumo (documento 1046/140 del 8 de Abril de 1997)

This Meeting has been declared to be an activity of *Interest for Public Health* by the Spanish Ministry of Health.(ref. 1046/140 of April 8, 1997)

PROGRAM OF *PSEUDOMONAS* '97

Thursday, September 4th

12.00 (noon). Registration

6.00 pm. Welcome by the Organizers and Authorities of the CSIC

6.30 pm. INAUGURAL LECTURE : **K. N. TIMMIS** (GBF, Braunschweig)
The thoroughly modern pseudomonad (Abstract I)

7.30 pm. Reception at the CSIC Headquarters

Friday, September 5th

WORKSHOP 1: ENVIRONMENTAL REGULATION OF CELL ACTIVITIES

5 Lectures: 40 min each (30+10)

Chairperson : C. Harwood, J. Mattick

9.00 am. **P. RAINEY** (U. Oxford)

Isolation and characterisation of Pseudomonas fluorescens genes induced solely on plant surfaces (Abstract II)

9.40 am. **V. DERETIC** (U. Michigan, Ann Arbor)

Regulation of mucoidy and stress response in Pseudomonas aeruginosa (Abstract III)

10.20 am. **D. OHMAN** (U. Tennessee)

Post-translational control of an alternate sigma factor involved in the expression of the alginate regulon in Pseudomonas aeruginosa (Abstract IV)

11.00 am. COFFEE BREAK AND POSTERS (#1-70) at the Patio of the Centro de Física
Miguel A. Catalán

11.40 am. **V. SHINGLER** (U. Umeå)

Sensing and responding to phenolic compounds by Pseudomonas CF600
(Abstract V)

12.20 pm. **J. GOLDBERG** (U. Virginia, Charlottesville)

Pseudomonas aeruginosa LPS expression in the environment of the host (Abstract VI)

1.00 pm. LUNCH AND POSTERS (#1-70) at the Patio of the Centro de Física
Miguel A. Catalán

SPECIALIZED SESSION : PSEUDOMONAS IN THE ENVIRONMENT

7 Presentations: 30 min each (25+5)

Chairperson : F. O'Gara, P. Lau

- 3.00 pm. **J. SPAIN** (Tyndall AFB, Florida)
Biodegradation of nitroaromatic compounds (Abstract VII)
- 3.30 pm. **S. MOLIN** (Technical University of Denmark, Lyngby)
Nondisruptive monitoring of bacterial consortia : A case of social life (Abstract VIII)
- 4.00 pm. **W. REINEKE** (U. Wuppertal)
Ortho- versus meta-pathway for the degradation of chloroaromatics (Abstract IX)
- 4.30 pm. **B. COSTERTON** (Montana State University, Bozeman)
Focused biodegradation by biofilm bacteria (Abstract X)
- 5.00 pm. **COFFEE BREAK AND POSTERS (#1-70)** at the Patio of the Centro de Física
Miguel A. Catalán
- 5.30 pm. **N. VAN DER LELIE** (VITO, Mol)
*Copper and lead resistance in *Alcaligenes eutrophus* CH34* (Abstract XI)
- 6.00 pm. **E. GALLI** (U. Milano)
Why o-xylene is so difficult to degrade : Dealing with a metabolic traffic jam
(Abstract XII)
- 6.30 pm. **N. ORNSTON** (Yale U., New Haven)
Contributions of coarse and fine recombination to metabolic evolution (Abstract XIII)
- 7.30 pm. **EVENING SHORT TALKS ON THE TOL PLASMID**
6 Presentations 15 min each (10+5)
Chairperson : V. Shingler
- P. WILLIAMS** (U. South Wales, Bangor)
*Characterisation and comparison of genes encoding 4-nitrotoluene catabolism in *Pseudomonas* sp. TW3 and 4NT* (Abstract XIV)
- W. DUETZ** (ETH, Zürich)
*Biodegradation kinetics of toluene, m-xylene, p-xylene and their intermediates through the upper TOL pathway in *Pseudomonas putida* (pWWO)* (Abstract XV)
- I. CASES** (CNB, Madrid)
Is the PTS system involved in the physiological coregulation of the upper TOL promoter? (Abstract XVI)
- S. MARQUES** (EEZ-CSIC, Granada)
*Activation and repression of transcription at the double tandem divergent promoters for *xylR* and *xylS* genes of the TOL plasmid of *P. putida** (Abstract XVII)
- N. KALDALU** (U. Tartu, Estonia)
Mapping of functional domains of the XylS protein (Abstract XVIII)

G. BERTONI (CSIC, Madrid)

Is the role of IHF at TOL Pu promoter purely architectural? (Abstract XIX)

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Saturday, September 6th

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WORKSHOP 2: SIGNAL TRANSDUCTION

5 Lectures: 40 min. each (30+10)

Chairpersons : J. Sokatch, S. Molin

9.00 am. **B. LUGTENBERG** (Leiden U.)

Towards the molecular basis of plant root colonization by Pseudomonas bacteria
(Abstract XX)

9.40 am. **J. MATTICK** (U. Queensland, Brisbane)

Regulatory pathways affecting the biogenesis and function of type 4 fimbriae in Pseudomonas aeruginosa (Abstract XXI)

10.20 am. **M. SCHELL** (U. Georgia, Athens)

New insights into the complex sensory network controlling virulence gene expression in the plant pathogen Ralstonia (Pseudomonas) solanacearum (Abstract XXII)

11.00 am. **COFFEE BREAK AND POSTERS** (#71-140) at the Patio of the Centro de Física
Miguel A. Catalán

11.40 am. **F. O'GARA** (U. College, Cork)

Gene regulation in the biocontrol strain Pseudomonas fluorescens F113 in response to environmental signals (Abstract XXIII)

12.20 pm. **P. CORNELIS** (U. Brussels)

Role of trehalose as a signal for the induction of antagonism against Pythium in P. fluorescens ATCC17400 (Abstract XXIV)

1.00 pm. **LUNCH AND POSTERS** (#71-140)

SPECIALIZED SESSION : CYSTIC FIBROSIS

7 Presentations : 30 min. each (25+5)

Chairpersons : D. Ohman, B. Costerton

3.00 pm. **A. LAZDUNSKI** (CNRS, Marseille)

Quorum sensing and production of virulence factors in Pseudomonas aeruginosa
(Abstract XXV)

3.30 pm. **N. HOIBY** (RHIMA Center, Copenhagen)

Chronic Pseudomonas aeruginosa infection in cystic fibrosis : a prototype biofilm infection (Abstract XXVI)

- 4.00 pm. **B. IGLEWSKI** (U. Rochester, NY) *Regulation of quorum sensing systems in Pseudomonas aeruginosa* (Abstract XXVII)
- 4.30 pm. **B. TÜMMLER** (Medizinische Hochschule, Hannover)
Differential analysis of genome organization and gene expression in Pseudomonas aeruginosa (Abstract XXVIII)
- 5.00 pm. COFFEE BREAK AND POSTERS (#71-140) at the Patio of the Centro de Física Miguel A. Catalán
- 5.30 pm. **F. BAQUERO** (Centro Ramón y Cajal, Madrid)
Population Biology and evolution of Pseudomonas bacterial lung colonisation in cystic fibrosis (Abstract XXIX)
- 6.00 pm. **T. NAKAZAWA** (Yamaguchi U.)
Production of virulence factors of Burkholderia cepacia : the regulatory gene cviR (Abstract XXX)
- 6.30 pm. **A. CHAKRABARTY** (U. Illinois, Chicago) *Intracellular signalling and regulation of growth and alginate synthesis in Pseudomonas aeruginosa* (Abstract XXXI)

7.30 pm. **ROUND TABLE ON PSEUDOMONAS GENOMICS**. Participants : **R. LEVESQUE** (U. Laval), **J. MATTICK** (U. Queensland, Brisbane), **B. TÜMMLER** (Medizinische Hochschule, Hannover), **J. LALUCAT** (U. Illes Balears)

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Sunday, September 7th

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WORKSHOP 3 : PROTEIN STRUCTURE, FUNCTION AND EVOLUTION

5 Lectures: 40 min. each (30+10)

Chairpersons : N. Ornston, P. Rainey

- 9.00 am. **L. ELTIS** (U. Laval)
Structure: function relationships in extradiol dioxygenases (Abstract XXXII)
- 9.40 am. **S. HARAYAMA** (Kamaishi City)
Protein evolution by DNA shuffling (Abstract XXXIII)
- 10.20 am. **K. FURUKAWA** (Kyushu University, Hakozaki)
Evolution of biphenyl dioxygenases in vitro by DNA shuffling for the degradation of PCBs and other biphenyl compounds (Abstract XXXIV)
- 11.00 am. COFFEE BREAK AND POSTERS (#141-210) at the Patio of the Centro de Física Miguel A. Catalán
- 11.40 pm. **R. DREW** (University College London)
Structural Studies of the P. aeruginosa Amidase Operon Regulatory Proteins

(Abstract XXXV)

12.20 pm. **R. PARALES** (U. Iowa, Iowa City)
Functional analyses of hybrid dioxigenases (Abstract XXXVI)

1.00 pm. LUNCH AND POSTERS (#141-210) at the Patio of the Centro de Física
Miguel A. Catalán

SPECIALIZED SESSION: BIOTRANSFORMATIONS

4 Presentations: 30 min. each (25+5)

Chairperson : S. Harayama, E. Galli

3.00 pm. **B. WITHOLT** (ETH, Zurich)
Effectiveness and cost of bioconversions of organic compounds: biological and physical bottlenecks (Abstract XXXVII)

3.30 pm. **J. DE BONT** (U. Wageningen) *Solvent-resistant Pseudomonas putida in the production of toxic fine chemicals* (Abstract XXXVIII)

4.00 pm. **C. HARWOOD** (U. Iowa, Iowa City) *How does Pseudomonas know what's out there? Chemotaxis and transport of aromatic compounds* (Abstract XXXIX)

4.30 pm. **A. STEINBÜCHEL** (U. Münster)
Polyhydroxyalkanoate biosynthesis in Pseudomonads and related bacteria : molecular analysis of the genes and utilization for production of polyesters in recombinant bacteria (Abstract XL)

5.30 pm. **DEPARTURE FOR EXCURSION AND DINNER IN SEGOVIA.** Please, make sure that you carry with you the invitation card, it will be requested at the bus.

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Monday, September 8th

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WORKSHOP 4: CELL SURFACES

5 Lectures: 40 min. each (30+10)

Chairperson : B. Lugtenberg, C. Harwood

9.30 am. **B. HANCOCK** (U. British Columbia, Vancouver)
Pseudomonas outer membrane proteins : Structure/function relationships (Abstract XLI)

10.10 am. **K. POOLE** (Queen's U., Kingston)
Pumps, pumps and more pumps : efflux-mediated multidrug resistance in Pseudomonas aeruginosa (Abstract XLII)

10.50 am. **H. SCHWEIZER** (Colorado State Univ., Fort Collins)

The essential Pseudomonas asd gene : its use as a selectable marker and derivation of attenuated mutants (Abstract XLIII)

11.30 am. COFFEE BREAK AND POSTERS (#141-210) at the Patio of the Centro de Física
Miguel A. Catalán

12.00 am. **R. DE MOT** (Leuven U.)
Cell envelope proteins and rhizosphere colonization of Pseudomonas fluorescens
(Abstract XLIV)

12.40 am. CLOSING LECTURE : **H. NIKAIDO** (U. California, Berkeley)
The resistance of Pseudomonas aeruginosa : Search for its molecular basis
(Abstract XLV)

LUNCH and DEPARTURES

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SPEAKERS' ABSTRACTS

I-XLV

The thoroughly modern *Pseudomonas*

Kenneth N. Timmis

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D-38124 Braunschweig, Germany

In 1986, J. Deshusses, J. Frey, S. Harayama and I organized an EMBO Workshop entitled "Genetic Manipulation of *Pseudomonas* - Applications in Biotechnology and Medicine" at the University of Geneva. This was the first of what became a tradition of *Pseudomonas* Workshops, which have since been held in Chicago (1989), Trieste (1991), Vancouver (1993), Tsukuba (1995) and Madrid (1997). In my presentation I shall reflect on how far the field has advanced in the decade following the first Workshop, and how our perception of this esteemed bacterium has changed.

In 1986 the term *Pseudomonas* was a catch-all for many obligately aerobic Gram negative bacteria which could not be confidently assigned to other groups. This meant on one hand that *Pseudomonas* had an identity problem but on the other that *Pseudomonas* researchers were a heterogeneous and gregarious group. Now a definitive taxonomy of *Pseudomonas* has been developed (e.g. ref. 1) and many organisms that *Pseudomonas* researchers worked on over the last decade have been expellited to groups with exotic names like *Burkholderia*, *Spingomonas*, *Ralstonia*, etc. All-of-a-sudden, *Pseudomonas* has changed from being inclusive to exclusive, and researchers who previously considered themselves *Pseudomonadologists* have to their chagrin discovered that they no longer belong to that erstwhile happy band. With hindsight it can be appreciated what foresight the early *Pseudomonas* meetings organisers had in creating the category "Honorary *Pseudomonads*" (2).

Another unwelcome realization relates to our perception of the ecological role and importance of *Pseudomonas*. On one hand, the persistent efforts of creative microbiologists to enrich and select bacteria capable of degrading or transforming exotic organic chemicals have been rewarded by the isolation of a wide range of new *Pseudomonads*, thereby reinforcing prevailing lab lore that they constitute some of the most versatile and prevalent microorganisms in soil and water. Direct analysis of microbial community composition by sequencing 16S rRNA genes cloned from DNA extracted from microbial communities indicates, however, that other types of microorganisms are generally numerically more significant, even in environments polluted with rich mixtures of exotic xenobiotics, the classical turf of *Pseudomonas* (3). Consistent with this is the finding that when *Pseudomonas* is introduced into a variety of habitats, it generally establishes a persistent population representing only about 0.01-1% of the total microbial community, irrespective of its initial concentration (4,5). While at this level *Pseudomonas* can and almost certainly does play an important functional role, our earlier perception of it ruling the microbial world was certainly misplaced.

In 1986 *Pseudomonas* was, possibly as a result of its identity problem, very promiscuous and apparently mated at embarrassing frequencies with practically anything in sight. Now, perhaps in response to an as yet undetected bacterial venereal disease, a new generation of more circumspect bacteria is developing which, by means of gene containment circuits, practices safer sex (6). The lethal event which ensues is reminiscent of mating in spiders, except that here it is the female which is the unlucky one.

If, in 1986, the sex life of *Pseudomonas* appeared somewhat chaotic, other aspects of its behaviour seemed simple. Gene regulation typically involved a regulatory protein which bound to its cognate recognition sequence in the promoter region of the gene regulated, and thereby stimulated or hindered RNA polymerase activity. It appeared to be a highly-localized, one dimensional affair. We now know that gene regulation may involve major contortions in

DNA structure (7, 8) which result in the precise positioning in three dimensional space of protein and DNA elements, some of which may be distantly separated from one another on the DNA molecule and, more seriously, that there is a whole management hierarchy (carbon control, growth phase control, etc.) involved in regulatory decisions which (at least in the cosseted environment of the test tube) frequently frustrate specific regulatory choices of the line management (9). This sharing of decision responsibility is not only a feature of the management hierarchy within the cell: populations of *Pseudomonas* communicate with one another and, when significant events require it, assemble a quorum able to take collective decisions (10) - a kind of bacterial *Landsgemeinde*.

Like all healthy democracies, *Pseudomonas* has developed strategic defences to ward off the assaults of others. In response to chemical warfare, it is able to regurgitate or neutralize various antibiotics (11) and projectiles (12), or to assume effective solvent resistant armour (13,14), and has developed sophisticated weaponry to defend its homelands - antibiotics against fungal invaders (15) and siderophores to starve its enemies (16). Its military skills have so far been most extensively studied in the context of *P. aeruginosa* pathogenesis, where the quality of its tactical weapons (toxins and exoenzymes) and their delivery systems (17,18), and its armour (19), have been elegantly exposed in minute detail.

On the other hand, *Pseudomonas* prefers to deal with problems, not by military action, but rather by seeking political solutions, preferably by forming strategic alliances with biotechnologists, to solve problems of the day, such as the degradation of environmental pollutants (20,21), plant growth stimulation (22) and plant protection (15), fossil fuel quality improvement (23,24), and so on, alliances which have so far yielded considerable mutual benefit and promise.

In summary, progress has furnished *Pseudomonas* with a clear identity but simultaneously relegated a lot of family members to the status of mere acquaintances. The estate has shrunk and enclosure fences have been erected. The biological cogs and wheels controlling the behaviour of *Pseudomonas* have been extensively characterized and this knowledge exploited to manipulate its behaviour in the laboratory and, to some extent, its behaviour once introduced for biotechnological purposes into the environment (20,25). Over the next decade we can expect the respectability of *Pseudomonas* to increase (and its charisma to decrease) as its financial worth, however, becomes recognized on Wall Street and its characteristic versatility systematically mined by a determined band of entrepreneurs. *Pseudomonas* is, however, a Jeckyl and Hyde character. As a star basking in the bright lights of the laboratory stage, *Pseudomonas* certainly seems very familiar to us. Its other life in the crowded jungles it inhabits as a shadowy minority member is less well known to us. Tracking it down in the real world and extracting its secrets is undoubtedly one of the major challenges of the next decade.

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Isolation and characterization of *Pseudomonas fluorescens* genes induced on plant surfaces

Paul B. Rainey

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Understanding the nature and function of microbial gene products expressed during growth *in vivo* is an essential step in attaining a molecular explanation for the "fit" between a bacterium and its environment. Genes likely to contribute significantly to "in vivo-fitness", and whose study may lead to an understanding of ecologically relevant traits, are those genes whose expression is activated solely in response to *in vivo* signals. Recent advances in gene fusion technology have resulted in the development of a genetic strategy termed *in vivo* expression technology (IVET) which provides a means of isolating bacterial genes that display elevated levels of expression *in vivo*. IVET is essentially a promoter trapping strategy which selects *in vivo* induced genes through their ability to drive the expression of a gene that is essential for survival *in vivo*. Random chromosomal fusions are generated upstream of a promoterless "in vivo-selected" marker in an autonomously replicating plasmid which is then integrated into the chromosome of the chosen host bacterium. Following *in vivo* selection, putative *in vivo*-induced genes are recovered from the bacterial chromosome for detailed molecular analysis. In order to achieve a molecular understanding of the ecology of plant-colonizing *Pseudomonas* we have developed two *P. fluorescens*-specific IVET strategies. The development and exploitation of these strategies will be described.

II

Regulation of Mucoity and Stress Response in *Pseudomonas aeruginosa*

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The genetic systems controlling conversion to mucoity in *P. aeruginosa* represent a paradigm for stress response in gram negative bacteria with universal implications for pathogenesis and physiology in a variety of bacterial species. A major pathway of conversion to mucoity is based on point mutations in the *algU mucABCD* gene cluster. Several lines of study regarding the nature of the genes within the *algU mucABCD* cluster have lead to the following conclusions of significance not only for the alginate system but also for bacteria in general:

(i) The *algU* gene encodes an alternative σ factor which is the founding member of a superfamily of σ factors referred to as ECF (extracytoplasmic function) or SEL (σ E-like) with a multitude of members among both gram negative and gram positive bacteria. A subset of the ECF (SEL) superfamily, the AlgU-RpoE family, encodes the equivalents of *P. aeruginosa* AlgU and RpoE from enteric bacteria. The AlgU-RpoE σ factors control extreme stress response in gram negative organisms.

(ii) The *mucA* gene encodes an anti- σ factor which inhibits AlgU function. MucA has equivalents and homologs among other members of the AlgU-RpoE family investigated so far and its mechanism of action serves as a model for understanding the mechanisms of control of the AlgU-RpoE σ factors.

(iii) The *mucB* gene encodes a periplasmic protein, conserved among the corresponding factors within the gene clusters encoding AlgU-RpoE sigma factors MucB negatively controls AlgU activity.

(iv) The *mucC* gene encodes a product with two predicted transmembrane domains and a CX₂CX₂CX₂C motif which are conserved among all MucC homologs. In combination with *mucA* or *mucB* mutations, *mucC* inactivation causes elevated levels of alginate production, suggesting its additive negative regulatory role in combination with *mucA* and *mucB*.

(v) The *mucD* gene encodes a homolog of the serine protease HtrA and other members of this family of proteins. Inactivation of *mucD* results in an overt mucoid phenotype. It has been proposed that MucD removes signals generated by environmental stress (e.g. denatured or oxidative damaged proteins) or otherwise affects AlgU activity.

Recent investigations of the status of *mucA* in a large collection of mucoid *P. aeruginosa* strains from cystic fibrosis patients indicated that the overall frequency of *mucA* alterations in clinical mucoid isolates exceeded 80 %. When tested in an animal model of respiratory infection, alginate overproduction by *mucA* *P. aeruginosa* improved their resistance to the innate clearance in the murine lung. Further analyses of the effects of *mucA* mutations on other systems in *P. aeruginosa* indicated additional implications regarding heat shock response of potential relevance for immunopathology in cystic fibrosis. Additional investigations using the yeast two-hybrid system and other means of examining relationships between the members of the *algU mucABCD* cluster lead to the current model of the regulation of AlgU applicable to other AlgU-RpoE σ factors.

III

Post-Translational Control of an Alternative Sigma Factor in the Expression of the Alginate Regulon in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa strains associated with cystic fibrosis are often mucoid due to the production of alginate, an exopolysaccharide and virulence factor. Alginate gene expression is transcriptionally controlled by a regulatory gene cluster at 68 min on the chromosome: *algT(algU)-mucABCD*. The *algT* gene encodes a 22 kDa ECF sigma factor (Sig22) the regulates its own promoter (PalGT) as well as the promoters of *algR*, *algB*, and *algD*. The other genes in the *algT* cluster appear to regulate the activity of Sig22. To better understand the interactions between Sig22 and its antagonist regulators, strain PAO1 was made to overproduce alginate (indicating high PalGD promoter activity) by increasing Sig22 in the cell via introduction of a plasmid clone containing *algT-mucA22*(defective) from mucoid strain FRD1. However, the bacterial cells remained nonmucoid if the clone included the next gene in the cluster, *algT-mucA22*(defective)-*mucB*, suggesting a stoichiometric relationship between Sig22 and MucB to control sigma activity. A Western blot analysis of total cell extracts showed that Sig22 was at least 10-fold higher in strains that overproduced alginate, even though *algT* gene expression increased less than 2-fold. This suggests that a post-transcriptional mechanism may destabilize Sig22 in order to control certain Sig22-dependent promoters. MucB was found to localize to the periplasm of the cell, whereas MucA localized to the inner membrane via one transmembrane domain. We propose a model in which Sig22-MucA-MucB interact via an inner membrane complex to control the stability of Sig22 protein in order to control alginate biosynthesis. In other studies, we identified the membrane-bound, cognate sensor histidine kinase (KinB) for the two-component regulator, AlgB. A carboxy-terminal fragment (C-KinB) was purified, and its ability to undergo progressive autophosphorylation *in vitro* was demonstrated. The phosphoryl label on C-KinB could then be rapidly transferred to purified AlgB. However, a *kinB::tn501* mutant generated in the FRD1 strain background retained the mucoid phenotype. Also, derivatives of AlgB with specific substitutions rendering it phosphorylation-defective were still capable of activating PalGD. Thus, although two-component regulators are typically activated by phosphorylation, this does not appear to be necessary for AlgB to fulfill its role in the transcriptional activation of PalGD.

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IV

Sensing and Responding to Phenolic Compounds by *Pseudomonas* CF600.

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Soil bacteria are capable of degrading a vast array of natural and synthetic aromatic compounds. To achieve this they encode specific enzymes which sequentially break down the compound to intermediates of central metabolism. The regulatory systems that control the expression of microbial aromatic catabolism have in general evolved to ensure that expression of the specialised catabolic enzymes is only undertaken when appropriate and sufficient substrate is present to provide a metabolic return. Thus, the regulatory systems may serve as the sensing mechanism of the bacteria for the presence of the compound, and their specificity and efficiency have the potential to limit the degradative capacity of a given biodegradative pathway.

We have sought to understand the sensing mechanism and limitations imposed by the regulatory circuit that controls expression of the methylphenol degradative pathway of *Pseudomonas* CF600, which encodes the regulated catabolism of phenol and some of its methylated derivatives via the *dmp*-system of a large megaplasmid called pVI150. The *dmp*-system is composed of the fifteen-gene *dmp*-operon and the closely linked but divergently transcribed *dmpR* regulatory gene. *DmpR* belongs to the α^{54} -dependent family of transcriptional activators. Unlike many other members of the family, the activity of *DmpR* is directly controlled, by interaction with distinct array of structurally related aromatic compounds, rather than via a sensory protein. Utilising hybrid proteins and effector response mutants of *DmpR* with altered aromatic effector activation profiles, we have been able to demonstrate that inefficient activation by *para*-substituted compounds is a major limiting factor for their catabolism. This information enabled us to generate strains that can degrade these compounds more efficiently. In addition, by introducing the broad-effector specificity *DmpR* regulator into another phenol degrading isolate, we have been able to expand the range of substrates that it can degrade.

The finding that the *DmpR* regulator is the sensory system of the bacteria and plays a substrate limiting role in its biodegradative ability led us to dissect the molecular mechanism underlying these properties. Data will be presented on how this regulator binds aromatics and thereby directly senses their presence in its environs, and on the molecular events that lead to unmasking of its transcriptional promoting property.

V

Pseudomonas aeruginosa strains exhibit different lipopolysaccharide (LPS) structures dependent on the site from which they were isolated. *P. aeruginosa* strains from the environment, from acute infections, and those initially infecting the lungs of cystic fibrosis (CF) patients are generally LPS-smooth, expressing long O side chain antigens. Strains isolated from chronic lung infections from CF patients are generally LPS-rough, with no, few, or short O side chain antigens (1). The molecular basis for the conversion to the LPS-rough form in the CF lung is not known, but apparently involves a stable mutational event, as LPS-rough isolates rarely convert to the LPS smooth form. We have previously shown that different CF isolates have different LPS mutations by demonstrating the ability of disparate genes from the O antigen locus to complement these mutations (2). We are currently comparing the nucleotide sequence of these genes from sequential isolates (3) recovered from early and later lung infections from the same CF patient. Previously, we have shown that the complete *P. aeruginosa* LPS core oligosaccharide is required for uptake into transformed human airway cells; structurally defined *P. aeruginosa* strains with an incomplete core and LPS-rough isolates from CF patients are taken up less well (4). This finding suggests that the LPS core of CF isolates may be incomplete; however, to our knowledge, the LPS structure has not been reported for any CF isolates. Studies are underway to determine the structural alterations in the LPS of chronically colonizing strains from CF patients. To begin a thorough analysis of the LPS core of *P. aeruginosa*, we have isolated genes encoding the enzymes required for the synthesis of the LPS core (the *rfa* genes). We have found that the *P. aeruginosa rfaF*, *rfaC*, *rfaG*, and *rfaP* genes are homologous to the corresponding genes in *Escherichia coli*. Sequence analysis also revealed that the translational start site for each gene overlapped with the stop site of the upstream gene, thus indicating that these *rfa* genes are likely part of a single operon. Studies to insertional inactivate each of these *rfa* genes and test these constructed LPS-core mutants for internalization into airway cells have been initiated.

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Nitroaromatic compounds can serve as growth substrates for bacteria. Either oxidative or reductive strategies can be involved in the initial attack that leads to the removal of the nitro group. Oxidative attack displaces the nitro groups of nitrobenzene, 2-nitrotoluene, dinitrotoluenes, and 3-nitrobenzoate. A multicomponent dioxygenase enzyme system, closely related to naphthalene dioxygenase, catalyzes the initial reaction that produces a catechol intermediate. To date strains able to degrade the above compounds have only been isolated from contaminated ecosystems and not from nearby uncontaminated areas. The evolutionary origin of the strains from a variety of sites is unknown. Comparison of nucleotide sequences reveals a striking similarity among the alleles that encode dioxygenase enzymes able to remove nitro groups. They are more similar to each other than to the corresponding naphthalene dioxygenase alleles. The preliminary evidence indicates a single origin for the genes and subsequent horizontal transfer.

The nitro groups of nitrobenzene, 4-nitrobenzoate, and 3-nitrophenol are reduced to the hydroxylamine level as the first step in the catabolic pathway. The pathway for nitrobenzene, which involves a series of unusual enzymes, seems to have evolved in response to an ecosystem where oxygen is limiting. The key enzyme, hydroxylaminobenzene mutase, seems unrelated to any other known enzyme based on comparison of the amino acid sequence to those in the database. The mutase converts hydroxylaminobenzene to amino phenol which serves as the substrate for ring fission catalyzed by an unusual dioxygenase related to extradiol ring fission enzymes. The ring fission product is oxidized in an NAD-dependent reaction and the amino group is removed in a hydrolytic reaction catalyzed by 2-aminomuconate deaminase. Subsequent steps are identical to those of the catechol meta ring fission pathway.

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We have investigated a toluene degrading bacterial community consisting of seven strains forming a complex three-dimensional structure after several days of growth in a flow chamber. Using quantitative rRNA *in situ* hybridization we could show that the linear correlation between growth rate and cellular ribosome concentrations often found in liquid monocultures for many bacteria also seems to be the rule for surface bound mixed communities. The reporter protein, Gfp, was introduced in order to monitor expression from fusions to the TOL promoters, Pm and Pu in a strain of *P. putida*. It was found that in flow chambers where benzylalcohol was supplied as the only carbon source the Pu promoter (induced by the substrate) was constitutively expressed, whereas the Pm promoter was only active if the reporter cells were attached to colonies of *Acinetobacter*. We finally followed the dispersal of a TOL plasmid in a biofilm, and the observation was that transfer only takes place after donor colonization of potential recipient colonies. Moreover, most plasmid carrying cells arise as a result of transconjugant proliferation rather than conjugation *per se*.

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The microbial degradation of various chloroaromatics has been described to occur via chlorocatechols as central intermediates, which are further degraded through the modified *ortho* pathway. Chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase fulfill the convergence of the chlorocatechol and catechol degradative pathways. Dechlorination takes place during cycloisomerization of chloromuconates and reduction of chloromaleylacetates. Alternatively to the intradiol type of ring cleavage the pathway is initiated by extradiol ring cleavage in some microorganism. The initial catechol 2,3-dioxygenases of the *meta* pathway are able to convert catechol, both isomeric methylcatechols, as well as 4-chlorocatechol at respectable rates. The further degradation of the ring cleavage product of 4-chlorocatechol seems to be a slow process since all strains degrading a chloroaromatic compound via 4-chlorocatechol through the *meta* pathway grow slowly on these substrates. However, when 3-chlorocatechol occurs in strains with a *meta* pathway the catechol 2,3-dioxygenase will be negatively influenced either by 3-chlorocatechol itself as a chelating compound resulting in a reversible inactivation or by a reactive acylchloride, the product of the cleavage of 3-chlorocatechol, which causes irreversible inactivation of the enzyme. Autoxidation of accumulating 3-chlorocatechol will lead to a general toxic effect onto the cells, and therefore degradation of haloaromatics via *meta* cleavage of 3-chlorocatechol was thought to be impossible.

Recently, *Pseudomonas putida* strain GJ31 was reported to degrade chlorobenzene with a generation time of 3 h via 3-chlorocatechol using the *meta* pathway without any apparent toxic effects. Data on the unusual *meta* cleaving enzyme that converts 3-chlorocatechol productively will be presented.

The dechlorination mechanism used by strain GJ31 represents an alternative to the dechlorination mechanisms which are normally used in the degradation of chlorobenzenes.

Bacteria living in natural environments grow preferentially in slime-enclosed biofilms adherent to available surfaces. In many instances bacteria adhere specifically to substrata that are also nutrient substrates. The act of adhesion to the surface concerned triggers a sweeping α factor - mediated phenotypic change in the bacterial cells and they may turn on or off the production of dozens of different structural proteins and/or enzymes. We must remember that the biofilm phenotype of a bacterium differs profoundly from the more commonly studied planktonic phenotype of the same species, and that we cannot extrapolate between these different forms of the organism in question. When bacteria have adhered to the surface, and begun the complex process of biofilm formation, each sessile bacterial cell is surrounded by the anionic polysaccharide of the biofilm matrix, and it therefore occupies a distinct microniche with sharply defined characteristics. Within this biochemically distinct microniche, the sessile bacterial cell is held in stable juxtaposition with relation to both the substratum and to its microbial neighbors. This stable spatial relationship between cells of the same or different species often leads to the development of functional consortia, in which all of the cells coordinate their biological activities, to produce quite startling levels of physiological efficiency. The stable juxtaposition of sessile cells vis-a-vis the substratum allows a very efficient "focused" attack on this surface, in which enzymes and other active molecules can bring their collective impact to bear on a remarkable small area. This very common phenomenon accounts for the tendency of a biofilm bacteria to cause local effects, such as "pitting", on surfaces that they have colonized.

In *Alcaligenes eutrophus* CH34 heavy metal resistance is encoded by its two megaplasmids, pMOL28 (180 kb) and pMOL30 (220 kb). Resistances to cadmium, zinc, cobalt (*czc* operon), mercury (*mer* of Tn4380), thallium, copper and lead are located on pMOL30. The genetic determinants encoding thallium, copper or lead resistance are not known. However, derivatives of pMOL30 with large deletions in the proximity of the *czc* region were often affected in lead and/or copper resistance, indicating that these resistances were probably encoded by two distinct determinants which are located near to *czc*. Also, it was shown that a Tn4431 insertion in the *czcS* gene affected lead resistance.

In order to study lead and copper resistance, a CH34 genomic bank was constructed in cosmid pLAFR3 and was introduced in the plasmid free, heavy metal sensitive strain *A. eutrophus* AE104. Subsequently, recombinant strains were screened for copper or lead resistance. Two independent cosmid clones encoding copper resistance were isolated which contained common DNA fragments. These clones were designated pMOL1023 and pMOL1024, respectively. Compared to pMOL30, pMOL1024 encoded full copper resistance (up to 1.5 mM), while the presence of pMOL1023 resulted in a lower but still significant resistance (1.0 mM, compared to 0.6 mM for strain AE104). A 21 kb region of pMOL1024, on which the copper resistance determinant (*cop*) was located, was sequenced and analysed. At least 8 ORFs were identified and were designated *copSRABCDGF*. The *copSR* genes encode a two component regulatory system, while the *copABCD* genes encode structural copper resistance genes which are similar to the *pcoABCD* and *copABCD* genes of *E. coli* and *P. syringae*, respectively. In contrast to the *E. coli* and *P. syringae* copper resistance operons, the regulatory genes *copSR* are transcribed in the opposite orientation to the structural genes *copABCD*. The ORF designated as *copG* showed similarity of amino acid sequence to protein W of the regulatory part of the CO₂ fixation operon of *Rhodobacter sphaeroides*. The *copF* gene, transcribed in the opposite orientation to *copABCD*, encoded a presumptive Cu-efflux ATPase similar to the PacS protein of *Synechococcus*. The similarity between CopF and other metal-ATPases will be discussed during this presentation. Analysis of pMOL30 and its deletion derivatives, as well as pMOL1023 and pMOL1024 indicated that expression of both *copABCD* and *copF* were required for full copper resistance.

A cosmid clone, designated pMOL1027, was isolated by complementation of strain AE104 for lead resistance. Subcloning and deletion analysis experiments allowed us to identify the region required for lead resistance. Sequence analysis of this region led to the identification of two ORF's, designated *pbrR* and *pbrA*. The *pbrA* gene is predicted to encode a protein with similarity to the CadA family of Cd-ATPases, and therefore presumably encodes a Pb-ATPase. Despite the similarity with CadA, no Cd resistance could be associated to *PbrA*. The *pbrR* gene, whose putative protein showed strong similarity with the MerR protein family, encodes the regulator of the *pbr* operon. This makes *PbrR* the first example of a member of the MerR regulator family that is involved in resistance to metals other than Hg. The *PbrR* protein has been overexpressed and its properties are being determined.

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Several soil bacteria can grow on toluene through different biochemical routes which have evolved among *Pseudomonads*. If toluene can enter many different pathways, the catabolism of xylenes appears more restricted. Several bacteria degrade *meta* and *para*-xylene through the progressive oxidation of a methyl group; *o*-xylene cannot enter this pathway and in the few bacteria so far described, this isomer appears to be oxidized *via* pathways involving either mono- or dioxygenation reactions of the aromatic ring without any processing of the methyl groups. *Pseudomonas stutzeri* OX1 is able to grow on toluene and *o*-xylene through two consecutive monooxygenations of the aromatic ring leading to the formation of mono- and dimethylphenols and methylcatechols respectively. The toluene/*o*-xylene monooxygenase (ToMO) is chromosomally encoded. A region coding for a phenol hydroxylase, and a catechol 2,3-dioxygenase has been also identified, suggesting that the toluene/*o*-xylene pathway could be organized in two operons which are divergently transcribed. From *P. stutzeri* OX1, M1 mutant was isolated, which had acquired the ability to grow on *m*- and *p*-xylene through the oxidation of a methyl substituent, but lost the ability to grow on *o*-xylene. From M1 strain a revertant strain R1 was further isolated, which grows on *o*-xylene and retains the ability to grow on *p*- and *m*-xylene. R1 strain degrades *o*-xylene through the direct hydroxylation of the aromatic ring, while the other two isomers are still degraded starting from the methyl substituent. The *o*-xylene and the *m*- and *p*-xylene catabolic pathways are both expressed in the strain R1, although on amount of the *m*- and *p*-isomers are transformed by ToMO into the corresponding dimethylphenols, which are unproductive for growth. Activation and inactivation of *P. stutzeri* OX1 and M1 methylbenzene catabolic routes has been shown to be mediated by a new 3 kb transposable element, which has been sequenced. The presence of this element in a region limited by *xyl* WC genes in OX1 leads to the failure of *m*- and *p*-xylene metabolism; its deletion in M1 strain allows the expression of *xyl* phenotype, while the inability of M1 to grow on *o*-xylene is due to the insertion of the same element in the region coding for the large subunit of ToMO (TouA). The R1 phenotype is consistent with the rearrangements observed which allow this strain to grow on *m* and *p*-xylene, even retaining a functional *o*-xylene catabolic pathway.

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One source of evolutionary variation is gradual change achieved by fixation of nucleotide substitutions in diverging genes. Relatively abrupt evolutionary alteration is achieved by genetic recombination, the placement of existing information in new contexts. Evolution sometimes demands swift change, and in these instances recombination is likely to be the predominant source of evolutionary diversity. Evidence for contributions of recombination emerges from discontinuities in evolutionary patterns as evidenced, for example, by rearrangement of homologous genes during their divergence in separate cell lines. Contributions of coarse recombination, rearrangement of relatively large fragments of genetic information, are generally recognized and are represented by the gene rearrangements that accompanied divergence of metabolic pathways in *Acinetobacter* and *Pseudomonas*. An additional example of coarse recombination is the process of gene conversion that contributes to genetic repair in *Acinetobacter*. Relatively short nucleotide sequences, ranging from 6 to 12 base pairs in length, contribute to fine recombination, processes whereby short nucleotide sequence repetitions may be gained by gene conversion or lost by deletion. Evidence for evolutionary contributions of fine recombination emerges from discovery of short nucleotide sequence repetitions in coding regions where selection is relaxed at the level of protein. The selective benefits afforded by fine recombination appear to emerge from the formation of sections of slippery DNA, and analysis of spontaneous mutations shows that the benefits conferred by such DNA can be counterbalanced by a predisposition to deletion.

Characterisation and comparison of genes encoding 4-nitrotoluene catabolism in *Pseudomonas* sp. TW3 and 4NT.

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The widespread use of nitroaromatic compounds in industry and their consequent release into the environment has led to their emergence as a major source of pollution. Bacterial strains capable of completely mineralising a wide range of nitroaromatics have been reported (1, 2). *Pseudomonas* sp. TW3 (3) and 4NT (4) are able to oxidatively metabolise 4-nitrotoluene via a route analogous to the 'upper pathway' of the TOL plasmids. We have determined the sequence and organisation of 5 genes from TW3, *nntWCMAB**, which are highly homologous to the *xyl* operon of TOL plasmid pWWO and obtained evidence that they encode enzymes responsible for the oxidation of 4-nitrotoluene and toluene to 4-nitrobenzoate and benzoate respectively (5). These genes share 84-99% identity at the nucleotide and amino acid levels with the corresponding *xyiWCMAB* genes and their products, additionally, *nntB** appears to be a pseudogene as it is interrupted by both a stop codon and a region of inserted DNA. We are currently characterising the activities of *nnt* gene products over-expressed in *E. coli* and investigating analogous genes which we have cloned from 4NT. We present preliminary data obtained from these recent studies.

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XIV

Biodegradation kinetics of toluene, *m*-xylene, *p*-xylene and their intermediates through the upper TOL pathway in *Pseudomonas putida* (pWWO).

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Pseudomonas putida mt-2, harbouring TOL plasmid pWWO, is capable of degrading toluene and a range of di- and tri-alkyl benzenes¹. Chemostat-grown cells ($D = 0.05 \text{ h}^{-1}$, toluene or *m*-xylene limitation) of this strain were used to assess the kinetics of the degradation of toluene, *m*-xylene, *p*-xylene, and a number of their pathway intermediates. The *v* vs *s* curves for the three hydrocarbons showed significant differences: the maximal conversion rates were rather similar ($11-14 \text{ mmol h}^{-1} [\text{g of dry wt}]^{-1}$) but the specific affinity (the slope of the *v* vs *s* curve near the origin) of the cells for toluene ($1300 \text{ liters. [g of dry wt]}^{-1} \text{ h}^{-1}$) was only 5% and 14% of those found for *m*-xylene and *p*-xylene, respectively. Consumption kinetics of mixtures of the hydrocarbons confirmed that xylenes are strongly preferred over toluene at low substrate concentrations. We also determined the maximum flux rates of pathway intermediates through the various steps of the TOL pathway as far as ring cleavage. Pulses of 3-methylbenzyl alcohol or 3-methylbenzaldehyde to fully-induced cells lead to the transient accumulation of 3-methylbenzoate. Accumulation of the corresponding carboxylic acid (benzoate) was also observed after pulses of benzyl alcohol and benzaldehyde, which are intermediates in toluene catabolism. Analysis of consumption and accumulation rates for the various intermediates showed that the maximal rates at which the initial monooxygenation step and the conversion of the carboxylic acids by toluate 1,2-dioxygenase may occur are 2-3 fold lower than those measured for the two intermediate dehydrogenation steps. The extent to which the two oxygenases of the TOL pathway limit the flux may be even stronger under natural conditions since oxygen is generally not present at air-saturated levels as in this study. Kinetic data such as presented here for the TOL pathway could be helpful in modelling the disappearance of the various BTEX compounds as a function of the size and composition of the BTEX degrading population. The a_A^0 values for various compounds may be of particular assistance in anticipating the residual concentrations that may be attained in time.

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Note: An oral presentation of this poster will be given at the micro-meeting on the TOL plasmid

XV

Is the PTS system involved in the physiological coregulation of the upper TOL promoter?

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The basis of the physiological regulation of the σ^{54} -dependent *Pu* promoter of the TOL (toluene biodegradation) plasmid pWW0 of *Pseudomonas putida* (1) have been investigated. The activity of *Pu-lacZ* fusions was silenced during exponential growth of *P. putida* in rich medium (2) as well as in minimal medium supplemented with casamino acids. In this medium, the level of β -galactosidase accumulation was modulated also (2-3 fold) by the addition of a supplementary carbon source, namely glucose or succinate. This suggested that catabolite control but also (and predominantly) growth phase down-regulate separately promoter activity. The loss of *ptsN*, a locus which maps closely downstream of *rpoN* and encodes a protein of the family of enzymes type-II of the phosphotransferase sugar transport (PTS) system (3), made *Pu* no longer responsive to repression by glucose, although the promoter was still silenced during rapid growth in rich medium. This indicated that growth phase control and carbon catabolite repression are two overlapping but genetically distinguishable mechanisms that inhibit *Pu* activity during rapid growth. The possible role of the *P. putida* PTS in the catabolite repression of *Pu* promoter will be discussed, based on the *ptsN* homology to the family of PTS enzymes type IIA specific for fructose and mannitol, and physiological data obtained in our laboratory.

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XVI

Activation and repression of transcription at the double tandem divergent promoters for *xyIR* and *xyIS* genes of the tol plasmid of *Pseudomonas putida*

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XyIR and XyIS regulators control transcription of the upper and *meta*-cleavage pathways for metabolism of toluene. The *xyIR* and *xyIS* genes are transcribed divergently and in the intergenic region four promoters are found. Pr1 and Pr2 are constitutive σ^{70} -dependent tandem promoters that drive expression of XyIR. The expression of *xyIS* gene is also driven from two promoters: Ps2 is a constitutive σ^{70} -dependent promoter, while 120 bp upstream the regulatable Ps1 promoter belonging to the σ^{54} class of promoters is positively regulated by XyIR. The architecture of Ps1 promoter is such that the UAS (XyIR targets) overlap σ^{70} consensus sequence at Pr1 and Pr2 promoters. Furthermore, in this region two sites for IHF binding are found, which are located at the -2/-30 and -136/-167 regions of Ps1 (*ie*, -6/-27 of Pr1 or +5/+20 of Pr2). Analysis of expression of mRNA from these promoters in different *P. putida* background bearing the TOL plasmid shows that expression of these four promoters is finely tuned through the interplay of these proteins in the 300 bp intergenic *xyIR-xyIS* region. XyIR protein has two functions in the regulation of the tol pathway. The protein is normally bound to its target sequence. This, in the absence of effector, slightly represses expression from Pr1 and Pr2, as proved by the fact that in a *xyIR* minus mutant, expression from these promoters increases at least ten times. In these conditions the level from Ps1 remains low, though detectable, while Ps2 is active. In the presence of effector, XyIR is known to change its pattern of interaction to its UAS; we show that a consequence of this change is a stronger repression of its own promoters, parallel with activation of transcription from Ps1. In these conditions, expression of Ps2 is not altered. In a *P. putida* background lacking σ^{54} , no expression occurred from Ps1, as expected. However, in the presence of effector, repression of both Pr promoters was such that mRNA was almost undetectable, suggesting that when no transcription was taking place at Ps1, clearance of XyIR at the UAS was almost negligible. Concomitant with this, expression from Ps2 was strongly induced, probably as a consequence of the lack of activity at Ps1. In an IHF minus background in the presence of effector, Ps1 was expressed at his highest level (more than ten times with respect to induced wild type strain levels). In contrast, the basal levels of this promoter were the lowest observed. No changes were observed in the expression of the three other promoters. An integrated model for the control of transcription from Pr and Ps promoters will be discussed.

XVII

The XylS protein activates the *P_m* (*meta*-cleavage) operon of the *P. putida* TOL plasmid which encodes a pathway for the catabolism of toluene, xylenes and related aromatic hydrocarbons. Substituted benzoates, the pathway intermediates, act as ligands of XylS and facilitate the site-specific DNA binding by the protein. We have generated two sets of overlapping deletions from both ends of the *xylS* gene to specify the parts of XylS which are responsible for DNA binding, oligomeric state, ability of transcriptional activation, and effector responsiveness. The truncated proteins were tested for solubility and stability. All truncated proteins which retained the 112 C-terminal amino-acids were capable to bind DNA and activate transcription from the *P_m* promoter. Thus, the C-terminus of XylS forms a DNA-binding domain which is sufficient for the activation of transcription. The 210 N-terminal amino-acids form a separable domain which provides the dimerization capability and ligand responsiveness.

The transcription initiation of σ^{54} -RNA polymerase (σ^{54} -RNAP) at TOL *P_u* promoter of *Pseudomonas putida* is regulated at a distance by the enhancer binding protein XylR in response to aromatic effectors such as toluene or xylene (1). *P_u* consists of a σ^{54} -RNAP binding site (-12/-24 region), a upstream activating sequence (UAS) for XylR binding located at about 100 bp 5' of the -12/-24 region, and an intervening sequence bearing a binding site for integration host factor (IHF) that is placed around -60 (2). The binding of IHF in the intervening sequence of *P_u* seems to be essential for promoter activity as shown by a *P. putida* mutant carrying an *ihfA* gene knockout that is not able to express the *upper* pathway operon for toluene degradation regulated from *P_u* promoter (3). Previous studies (4) showed that the DNA-bending protein IHF plays a clear architectural role in *P_u* assisting the looping out of the intervening sequence and allowing the contact between XylR and σ^{54} -RNAP, both pre-bound about 100 bp apart. IHF may play, however, additional roles. Data obtained with Dnase I footprinting and gel retardation will be presented that support the notion that IHF assists the recruitment of the σ^{54} -RNAP at -12/-24 region.

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Plant roots are colonized by microbes which are supposed to grow on exudate compounds. Among these microbes are soil-borne pathogens which cause diseases. The process of root colonization can be applied for beneficial purposes such as biocontrol, biofertilization, bioremediation and phytostimulation. In attempts to identify bacterial genes and traits involved in root colonization we have used two approaches. By predicting possible colonization traits and subsequently testing mutants impaired in that trait on their ability to colonize in competition with the parental strain, we have identified the following colonization traits: the syntheses of flagella, the O-antigen of LPS (lipopolysaccharide), amino acids and vitamin B1 as well as a high growth rate and utilization of major exudate carbon sources. Using another approach, we tested random Tn5(*lacZ*) mutants on their competitive colonization ability and found that mutations in the following genes resulted in impaired colonization. (i) A two-component system (*colR/S*) of which neither the stimulus nor the trait has been identified yet. (ii) *ssx*, encoding a site-specific recombinase belonging to the lambda integrase gene family. (iii) *nuo*, which in *E. coli* consists of a 14-gene operon encoding NADH: ubiquinone oxidoreductase, one of two enzymes involved in the generation of the proton motive force.

ACKNOWLEDGMENTS

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Type 4 fimbriae (pili) are virulence factors which promote attachment of pathogenic bacteria to epithelial tissue and mediate a form of flagella-independent surface translocation called twitching motility (TM). In *Pseudomonas aeruginosa* over 30 genes involved in fimbrial biogenesis have so far been identified, which show that type 4 fimbriae are a subset of a supersystem involved in assembling related cell surface complexes adapted to different purposes, including protein secretion and DNA uptake, in a wide variety of bacteria (1). There are also at least three regulatory pathways which control fimbrial assembly and function. The first is a classic two-component σ 54-dependent sensor-regulator system, encoded by *pilS* and *pilR*, which controls the transcription of the fimbrial subunit gene *pilA*. (2). The second, encoded by *fimS* and *algR*, defines a new class of sensor-regulator / transmitter-receiver systems which appear to involve an anti-sensor capable of toggling the receiver from one state to another to control different pathways, in this case twitching motility and alginate synthesis (3).

The third system, involving a large cluster of genes *pilGHIJKL* and *chpA/B*, represents the most complex signal transduction pathway yet described in prokaryotes, and which appears to regulate multiple inputs and outputs via a central transducer with 6 phosphorylation domains. This system shows multiple similarities to *che* genes which effect the chemotactic control of flagellar rotation in enteric bacteria, and to *fzr* genes which control gliding motility in *Mycococcus vanthus*, the latter of which appears to be closely related to twitching motility. The genes *pilGHIJK* were defined by A. Darzins (4) and we have now identified the downstream genes in this cluster: *pilL*, *chpA* and *chpB*. *ChpB* (343 a.a.) is homologous to the MCP-methyltransferase protein CheB, and on this basis would be expected to demethylate the MCP-like protein PilJ. PilJ (836 a.a.) has slight N-terminal sequence similarity to the autophosphorylation site of the phosphotransmitter protein CheA but no other obvious homology to other genes/proteins in the databases, while *ChpA* (1638 a.a.) has extensive similarity to CheA. An unusual feature of *ChpA* is the occurrence at its N-terminus of six repeats, each of which contains a possible autophosphorylation site, suggesting that this protein can have multiple phosphorylation states. At its C-terminal end *ChpA* is homologous to the response regulator / receiver CheY. PilG and PilH are also homologous to CheY. Thus *ChpA* appears to be at the centre of a complex phospho-transfer system, in which signals received from PilJ and possibly other sources are relayed either by intramolecular phosphotransfer and/or by phosphorylation of ChpB, PilG or PilH, and possibly other receivers, presumably to integrate multiple inputs and to effect multiple responses, one of which is twitching motility.

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New insights into the complex sensory network controlling virulence gene expression in the plant pathogen *Ralstonia* (*Pseudomonas*) *solanacearum*.

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Ralstonia (*Pseudomonas*) *solanacearum* causes a lethal wilting disease of over 200 different plants all over the world. It secretes plant cell-wall-degrading exoenzymes and the exopolysaccharide EPS I, both of which are critical for successful colonization of the vascular system of a host after root invasion. Production of large amounts of EPS I is also a primary cause of wilting (and killing) of infected plants due to its ability to block water flow in the xylem. EPS I is a >1000 kDa linear polymer comprised of 3 uncommon amino sugars; biosynthesis and export of EPS I are encoded by the 16-kb *eps* operon. Transcription of the *eps* operon and other virulence genes (*e.g.* those encoding some exoenzymes) is controlled by a large interactive regulatory network comprised of at least 10 proteins and responsive to multiple environmental signals. The network is comprised of two distinct two-component regulatory systems (VsrAD and VsrBC), the unique signal integrator protein XpsR, and the unusual PheC signal transduction system. The PheC system appears to encode a phosphorelay cascade that regulates activity of PheA, a LysR-type transcriptional regulator which globally controls many virulence genes and regulates transition of *R. solanacearum* from a soil saprophyte into a virulent plant pathogen. Evidence will be presented that activity of the PheC system is controlled by a new type of volatile, quorum-indicating molecule, 3-OH palmitic acid methyl ester. Recent investigations of the protein-DNA and protein-protein interactions that occur between network components to allow control of *eps* transcription in simultaneous response to 3 independent signals will also be presented. Finally, the recent discovery and analysis of new target genes regulated by the network will be discussed. Among these is a LuxR/LuxI-type autoinducer system, suggesting that *R. solanacearum* has a two-part hierarchical quorum sensing system.

XXII

Regulation of the biocontrol metabolite 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* F113

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The biocontrol ability of *P. fluorescens* F113 is conferred through the production of the potent antifungal metabolite 2,4-diacetylphloroglucinol (Phl). Detailed analysis of a Phl biosynthetic locus previously identified and isolated from the F113 genome was completed. Characterisation of the biosynthetic locus by sequence analysis identified at least four putative open reading frames involved in the biosynthesis of Phl and a divergently transcribed locus-linked negative regulator specific for the repression of Phl biosynthesis. This putative repressor shows similarity to other well characterised repressors including TetR.

Our recent studies have largely focused on the role of this putative repressor on Phl biosynthesis. Addition of multicopies of the putative repressor to the F113 wildtype significantly reduced Phl production. This effect was shown to be mediated at a transcriptional level using a Phl biosynthetic gene::lacZ fusion. Expression of Phl in F113 is growth phase dependent. Inactivation of the repressor gene (*phlR*) in F113, resulted in constitutive Phl production. A truncated clone, from which the putative repressor was removed exhibited constitutive Phl production in the wildtype and Phl biosynthetic mutant backgrounds. Removal of the Phl negative regulator also decouples Phl synthesis from *ApdA/GacA* two component regulator control.

Phl production was conferred to all heterologous *Pseudomonadaceae* backgrounds assessed upon introduction of the truncated clone. This offers the potential for the construction of novel GMO's expressing multiple biocontrol traits.

XXIII

Role of trehalose as a signal for the induction of antagonism against *Pythium* in *P. fluorescens* ATCC 17400

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Pseudomonas fluorescens ATCC 17400 shows an *in vitro* antifungal activity against *Pythium debaryanum* in conditions of iron limitation. A mini-Tn5- β galactosidase transposition mutagenesis in *P. fluorescens* ATCC 17400 allowed the identification of clones producing β -galactosidase only in the presence of *Pythium* culture supernatant. Using an inverse PCR approach, the Tn5 insertion was found to be in the trehalase gene (*treA*), as judged by similarity search with the data bank (1). Indeed, the induction of the *lacZ* gene was lost upon treatment of the fungus supernatant with commercial trehalase while trehalose concentrations as low as 1 μ M could induce the *treA-gal* fusion. The mutation in *treA* had no effect on the *in vitro* antagonism, but drastically decreased the osmotolerance of the mutant in liquid culture, and suppressed the ability of *P. fluorescens* ATCC 17400 to utilize trehalose as a carbon source. A second transposon mutagenesis was done in the *treA* mutant in order to find mutants which had lost the trehalose-induced β galactosidase production. Such a mutant was found with an insertion in a *treP* homologue, a trehalose phosphotransferase gene (1). This double mutant could not anymore restrict fungal growth, except under conditions of high osmolarity. The same conditions were also found to increase the antagonism of the wild type. A PCR amplification was done using primers corresponding to the sequences flanking the two transposons and a 2 kb fragment was obtained and sequenced. This fragments contains two *orf*, one corresponding to the trehalase gene, *treA*, the other one to the trehalose phosphotransferase gene, *treP* (1). These data confirm the role of the disaccharide trehalose in osmotolerance in *P. fluorescens*, and indicate an additional role as a signaling molecule initiating a transduction pathway leading to fungus antagonism.

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XXIV

Quorum sensing and production of virulence factors in *Pseudomonas aeruginosa*

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A continually increasing number of quorum sensing systems are being identified in different species. LasR-LarI was the first to be identified in *Pseudomonas aeruginosa* and has been shown to regulate the expression of *lasB*, *lasA*, *aprA* and *toxA*. The second was RhlR-RhlI, which was primarily identified as a regulator required for rhamnolipid synthesis but also influences elastase synthesis. While investigating several different phenomena, we independently cloned and characterized two regulatory genes, *vsmR* and *vsmI*, in strain PAO1 (1). Ces genes were shown to be identical to *rhlR* and *rhlI*, identified in strain PG201. Besides rhamnolipid and elastase, *vsmR* has an extended regulatory impact, being involved not only in regulation of a second protease (*AprA*) but also of secondary metabolites such as cyanide and pyocyanin (1). It was shown that VsmI directs the synthesis of N-butanoyl-L-hexanoyl-homoserine lactone (BHL) and of N-hexanoyl-L-homoserine lactone (HHL) (2). We also showed that a hierarchical quorum-sensing cascade links the transcriptional activators LasR and VsmR to expression of the stationary-phase sigma factor RpoS (3). In addition to *vsmI*, *rpoS* and several genes encoding exoproducts such as staphylytic enzyme, lectin and chitinase, the *xcp* genes (encoding the Xcp secretion machinery) have recently been shown to be regulated by quorum sensing (4). We also have some evidence that additional regulatory genes affect *vsmR* expression. Undoubtedly, additional studies on the various levels of regulation of *lasR* and *vsmR* will help our understanding of the *P. aeruginosa* quorum sensing.

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XXV

Chronic *Pseudomonas aeruginosa* infection in cystic fibrosis: a prototype biofilm infection.

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Cystic fibrosis (CF) is a genetic heterogeneous disease, which is transmitted as an autosomal recessive trait. The CF gene product is a membrane-bound protein called the CF Transmembrane Conductance Regulator (CFTR) Protein. This protein is the chloride ion channel which regulates the transportation of chloride ions across fluid-transporting epithelial cells. The CF defect of the CFTR protein leads to altered secretions (salty sweat, thick mucus), blocked ducts and thereby reduced mucosal defense which, in turn, leads to recurrent intermittent and chronic bacterial infections in the lungs caused by *S. aureus*, *H. influenzae*, *B. cepacia* and *P. aeruginosa*. Intermittent colonization is caused by non-mucoid *P. aeruginosa*, whereas chronic infection is caused by mucoid (alginate producing) *P. aeruginosa* forming typical biofilms in the bronchioles and bronchi without ever giving rise to systemic infection. The acute or chronic bacterial infections lead to recruitment of the inflammatory defense mechanisms of the lungs such as IgG and polymorphonuclear neutrophil leucocytes (PMNs). Already in CF infants, therefore, low grade inflammation is detectable. In case of overt pulmonary infection, the inflammation gives rise to further tissue damage notably by release of elastase and DNA from PMNs. If the infection is not eradicated and becomes chronic, then immunocomplex mediated tissue damage continues dominated by PMNs. It is possible to prevent or at least delay the onset of the chronic *P. aeruginosa* biofilm infection by early aggressive therapy of the intermittent colonization with oral ciprofloxacin in combination with colistin inhalation for 3 weeks. A rising titer of antibodies against e.g. *P. aeruginosa* indicates the onset of the biofilm formation and chronic infection and guides the decision to start maintenance antibiotic therapy since eradication of the biofilm infection is not possible. The principle is to restore lung function repeatedly by regular 2 week courses of intensive intravenous treatment every 3 months in the CF centre and daily inhalations of colistin between the courses of intravenous antibiotics and sometimes also by giving oral ciprofloxacin during these intervals. Inhalation of budesonide and recombinant DNase is also used by most patients. The main result of the aggressive treatment is a probability of 83% of surviving 40 years after the diagnosis of CF in the Danish centre.

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Regulation of and by *Pseudomonas aeruginosa*: The Quorum Sensing Systems LasR-PAI-1 and RhlR-PAI-2

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Expression of growing number of *Pseudomonas aeruginosa* virulence factor genes such as *lasA* and *lasB* elastase, *aprA* (alkaline protease) and the rhamnolipid genes *rhlA* and *rhlB* has been shown to be regulated in a cell-density-dependent fashion termed quorum sensing. *Pseudomonas aeruginosa* has two distinct quorum sensing systems: LasR-PAI-1 and RhlR-PAI-2. Each of these systems has been shown to activate transcription of many genes in a hierarchical fashion. Thus LasR-PAI-1 preferentially activates *lasI* over *lasB* and RhlR-PAI-2 activates *rhlAB* preferentially over *lasB*. Expression of the quorum sensing genes *lasR*, *lasI*, *rhlR* and *rhlI* and their protein products is also highly controlled. Expression of *lasR* absolutely requires Vfr (the *Pseudomonas aeruginosa* homologue of *E. coli* CAP) and *lasR* expression is enhanced by LasR-PAI-1. The *lasI* and *rsdA* genes are activated by LasR-PAI-1 and *lasI* expression is negatively regulated by RsaL. The LasR-PAI-1 system is dominant over the RhlR-PAI-2 system. Expression of *rhlR* is enhanced by LasR-PAI-1. Furthermore, PAI-1 can compete with PAI-2 for binding to RhlR thus preventing the activation of RhlR. Thus Las-PAI-1 regulates the *rhlR* systems at the transcriptional and post-transcriptional levels. Expression of *rhlI* is activated by Rhl-PAI-2 and it is negatively regulated by a distinctly separate protein. Mutations in the quorum sensing system genes reduces the virulence of *Pseudomonas aeruginosa* in a neonatal mouse model of acute pneumonia.

Differential analysis of genome organization and gene expression in
Pseudomonas aeruginosa

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Genomic diversity of *Pseudomonas aeruginosa* was assessed by macrorestriction and genetic mapping of the chromosomes (1, 2). Southern hybridizations of PFGE-separated *SpeI*-restricted chromosomes with genes (2, 3), linking probes (2) and *SpeI* fragments of strain PAO cloned in YACs, high resolution restriction mapping of *SpeI* fragments, PCR-assisted subtractive genome hybridization (see K. Schmidt et al., this meeting) and by sequencing of six loci approximately equidistantly distributed on the PAO chromosome (see C. Spangenberg et al., this meeting). The number of 4 *rrn* operons, but not the 1-*CeuI* backbone, was conserved in all analyzed strains. The circular chromosomes varied between 5.2 and 7 Mb in size (2). Intra- and interclonal mapping of PAO and clone C strains (2, 4) revealed a mosaic structure of the genome. The acquisition and loss of blocks of DNA appear to be the major factor for genome diversity in *P. aeruginosa*. Comparative analysis of clone C isolates from various habitats uncovered the exchange of DNA in up to 10% of the chromosome. However, in general the gene order was conserved. Three genomic regions encoded in strain PAO by *SpeI* fragments SpW-SpAA, SpU and SpV were probed with YACs. Of 97 analyzed strains representing 47 genotypes, the sequence contigs at the supragenomic level of 100 kb were maintained in more than 98% of cases. Two complex genome rearrangements involving either multiple insertions and deletions or truncation and transpositions were resolved by Smith-Birnsteil mapping with YAC fragment end probes. Sequencing of 19 strains in six loci gave the result that codon position and local sequence environment determine the rate of nucleotide substitutions. The frequency of single nucleotide polymorphisms within genes is unrelated to the global plasticity of the adjacent genomic region and its macrorestriction fragment length diversity. In contrast to many other bacterial species, the clonal population of *P. aeruginosa* exhibits no significant linkage disequilibrium between distant loci.

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XXVIII

Population biology and evolution of *Pseudomonas* bacterial lung colonisation in
cystic fibrosis

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ABSTRACT NOT SUBMITTED

XXIX

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Human infections with the phytopathogen *Burkholderia cepacia* occur relatively infrequently and are usually nosocomial and affect immunocompromised patients. However, the frequency of isolation of *B. cepacia* from the sputum of cystic fibrosis patients is increasing. Despite of its evolving role in pulmonary infection in CF patients, very little is known about the pathogenesis of *B. cepacia*. We analyzed a nosocomial outbreak of *B. cepacia* and revealed that a majority of isolates in a single hospital are derivatives of a single strain, although they have various biochemical characteristics(1). One of the strains, JN106, produces a hemolytic substance, cepalydin, as well as protease, lecithinase, and lipase. Cepalydin is a small hydrophobic compound with potent antifungal activity (2). Protease of JN106 is the size of 37-kDa and immunogenically similar to the protease produced by *B. cepacia* KFI and strains from independent sources (3). Transposon mutagenesis and subsequent cloning of the disrupted gene were carried out on JN106 by the established procedure (4). A cepalydin- and protease-negative mutant JNT1061 had a mutation in an open reading frame for a 327 amino acid polypeptide belonging to LysR family proteins. A typical helix-tern-helix motif was found in the N terminal region. Protease activity recovered completely when the *lysR* family gene termed *cviR* (putative regulator of cepacia virulence) on a plasmid. Cepalydin activity was also restored, though a little, suggesting that the *cviR* gene is involved in the production of both protease and cepalydin. Lipase and lecithinase activities as well as motility and growth on L agar were not affected by the mutation. Southern hybridization with a *cviR* probe revealed that the gene is ubiquitous among *B. cepacia* strains independently to the origin of the bacteria and to the production of cepalydin and protease. The lethality of mice by intratracheal challenge of *B. cepacia* showed a significant difference between the JN106-challenged and the JNT1061-challenged groups, suggesting a potential role of cepalydin and/or protease in the pathogenicity of *B. cepacia*.

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XXX

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Pseudomonas aeruginosa is a major pathogen in the lungs of cystic fibrosis patients where it becomes heavily mucoid during prolonged infection due to production of an exopolysaccharide called alginate. Alginate is believed to protect the infecting cells from phagocytosis and antibiotic therapy, thereby contributing to their virulence. *Pseudomonas aeruginosa* is also known to undergo a transition to mucoidy during prolonged growth under conditions of nitrogen or phosphate starvation, or in presence of inhibitors of energy metabolism. To understand how starvation for phosphate or low energy levels within the cells act as triggers for alginate synthesis, we have defined the role of an enzyme nucleoside diphosphate kinase (Ndk), which is normally responsible for the synthesis of nucleoside or deoxynucleoside triphosphates (NTP/dNTP), on alginate synthesis. Alginate synthesis requires as precursors phosphorylated sugars and GTP to produce GDP-mannose, an essential precursor of alginate. We have demonstrated that GTP synthesis in *P. aeruginosa* occurs predominantly in the late exponential or early stationary phase due to formation of complexes of Ndk with other proteins such as pyruvate kinase (P_k), a Ras-like protein Pra, or elongation factor Tu (EF-Tu). High levels of GTP give rise to (p)ppGpp through the action of a ribosome-associated enzyme RelA (and another enzyme SpoT), the accumulation of which leads to inhibition of the degradation of another intracellular macromolecule polyphosphates (polyP). A single mutation in the *algR2* gene of a mucoid strain 8830 leads to an extremely low level of alginate, polyP and ppGpp whose formation is restored by introducing or hyperexpressing the *algR2* or the *ndk* gene in the *algR2* mutant. Thus *ndk* plays a major role in the synthesis of GTP, ppGpp, polyP and alginate. Since both GTP and ppGpp are signalling molecules, and since in *Escherichia coli*, ppGpp is known to activate the stationary phase sigma factor RpoS and polyP is known to be important for cell survival in the stationary phase, and since in *Pseudomonas aeruginosa*, alginate is produced primarily at the onset of the stationary phase, the role of various Ndk complexes with membrane-associated proteins such as P_k and Pra which provide GTP at the onset of the stationary phase, will be discussed.

XXXI

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Extradiol dioxygenases catalyze the *meta*-cleavage of catechols. These metalloenzymes play an important role in determining the specificities of many microbial aromatic catabolic pathways including those responsible for the aerobic degradation of toluates and biphenyl/PCBs (*bph*). Efforts in our laboratory are directed towards (i) understanding the molecular mechanisms of this important class of enzymes, and (ii) engineering extradiol dioxygenases with bioremedially useful properties. With the insights gained from high resolution crystal structures of 2,3-dihydroxybiphenyl dioxygenase (DHBD), the extradiol dioxygenase of the *bph* pathway, the classification of extradiol dioxygenases has been expanded, structural determinants of specificity have been tentatively identified and the catalytic roles of conserved active site residues have been proposed. To further investigate the structural determinants of specificity, several variants of DHBD have been prepared by oligonucleotide-directed mutagenesis. The effect of the substitutions on the steady-state kinetic parameters (*K_M* and *k_{cat}*) of the anaerobically purified variants as well as the stability of their respective ferrous catalytic centres has been determined for a variety of substrates. Complementing this approach, the specificities of several DHBD isozymes have been compared.

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The use of enzymes in medical, industrial and environmental purposes is prevalent today, and will continue to expand rapidly in the next century. Traditionally, the selection of useful enzymes to be incorporated into commercial applications has been done by screening enzymes or microorganisms adapted to desired environments. The emergence of protein engineering technologies exemplified site-directed mutagenesis and computer-assisted modeling allowed completely new approaches to obtain novel enzymes. However, many attempts for specifically altering the properties of enzymes by this approach failed because introduced amino acid substitutions exerted unexpected effects on the structure and function of the target enzymes. In order to achieve a number of improvements of potential commercial benefit, other methods than "the rational design of new enzymes" are also desired. One of the possible ways to do it is to perform an extensive mutagenesis in combination with a shuffling of protein sequences. We examined the possibility of improving catechol 2,3-dioxygenases via the shuffling of the protein sequences. Catechol 2,3-dioxygenases encoded by *xyIE* and *nahH* are 84% identical in their amino acid sequences. A set of *nahH-xyIE* hybrid genes was constructed, and hybrid catechol 2,3-dioxygenases produced from the *nahH-xyIE* hybrid genes were purified along with two natural enzymes, *XyIE* and *NahH*. Some of the hybrid catechol 2,3-dioxygenases were much stable than the natural enzymes at high temperatures. This observation suggested that chimeric analogs derived from pairs of homologous proteins could gain unexpected functions. We then developed a new PCR-based method to isolate diverse gene sequences encoding catechol 2,3-dioxygenases from environmental samples such as soil and seawater. Using the isolated gene sequences encoding catechol 2,3-dioxygenases, DNA shuffling was carried out. The properties of catechol 2,3-dioxygenases synthesized from the shuffled genes will be discussed.

Evolution of biphenyl dioxygenases *in vitro* by DNA shuffling for the degradation of PCBs and other biphenyl compounds

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The biphenyl dioxygenases (BP Dox) of strains *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia cepacia* LB400 exhibit distinct difference in the substrate range of PCB despite nearly identical amino acid sequences. The range of congeners oxidized by LB400 BP Dox is much wider than that oxidized by KF707 BP Dox. The PCB degradation abilities of these BP Dox were highly dependent on the recognition of the chlorinated rings and the sites of oxygen activation. The KF707 BP Dox recognized primarily the 4'-chlorinated ring (97 %) of 2,5,4'-trichlorobiphenyl, and introduced O₂ at the 2',3' position. The LB400 BP Dox recognized primarily 2,5-dichlorinated ring (95%) of the same compound, and introduced O₂ at the 3,4 position. It was confirmed that the BphA1 subunit (iron-sulfur protein of terminal dioxygenase encoded by *bphA1*) plays a crucial role in determining the substrate selectivity. We constructed a variety of chimeric *bphA1* genes by exchanging four common restriction fragments between the KF707-*bphA1* and the LB400-*bphA1*. Observation of *E. coli* cells expressing various chimeric BP Dox revealed that a relatively small number of amino acids in the carboxy-terminal half are involved in the recognition of the chlorinated ring and the sites of dioxygenation, thereby are responsible for the degradation of PCB.

We then constructed a hybrid strain KF707-D34 which shows wide degradation capability of PCB and other biphenyl compounds. This strain was originated from KF707 and possessed a chimeric *bphA1*, in which only a small part of *bphA1* sequence was replaced from LB400. The gene targeting replacement was achieved by homologous recombinations within the chromosomal KF707 *bph* operon. The strain KF707-D34 degraded 4,4'-CB via 2,3-dioxygenation (100%), 2,5,4'-CB via 3,4-dioxygenation (96%) of 2,5-dichlorinated ring along with via 2,3-dioxygenation (4%) of 4'-chlorinated ring and 2,5,2',5'-CB via 3,4-dioxygenation (100%). Sequence analysis of KF707-D34 *bphA1* revealed that 4 nucleotides were changed from the KF707 *bphA1* gene to those of the LB400 *bphA1* gene, resulting in only one amino acid substitution of Thr to Asn at the position 376.

We have obtained more combinations of chimeric *bphA1* genes by *in vitro* shuffling by using PCR. *E. coli* strains expressing these chimeric BP Dox exhibited various degrees of activities for biphenyl compounds.

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XXXXIV

Structural Studies of the *Pseudomonas aeruginosa* Amidase Operon Regulatory Proteins

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Amide dependent expression of the catabolic amidase operon of *P. aeruginosa* occurs via a simple signalling pathway. Initially, inducing amides interact with AmiC the ligand sensor protein and the signal is then transduced to the response regulator AmiR, an RNA binding protein and transcription antitermination factor. AmiR interacts with specific sequences in the constitutively produced operon leader mRNA transcript and prevents formation of an upstream *rho*-independent transcription terminator. This leads to the production of full length operon transcripts. It would appear that a simple steric hindrance mechanism controls the regulatory system since over-expression of amiC leads to a non-inducible amidase phenotype and over-expression of amiR leads to constitutive amidase expression.

AmiC is structurally related to the periplasmically located branched-chain amino acid binding protein family with two domains surrounding a central cleft. The crystal structures of wild type AmiC with acetamide (the on configuration), and wild type AmiC with butyramide (the off configuration) have been determined. AmiC with acetamide adopts a completely closed down structure whereas binding of the co-repressor butyramide to AmiC leads to a slight opening of the ligand binding cleft. The crystal structure of a 'butyramide inducible' AmiC mutant with butyramide has also been determined and shown to be a completely closed down form. A complex of wild type AmiC with AmiR has been isolated in the presence of butyramide composed of an AmiC dimer and an AmiR dimer (Mr 128,000). Gel filtration analysis has shown that the AmiC/AmiR complex dissociates upon the addition of acetamide confirming the steric hindrance model as the mechanism of amide dependent induction of the amidase operon. The use of a periplasmic binding protein fold to sense ligands and modulate the activity of an RNA binding protein is as far as we are aware unique.

XXXXV

Aerobic degradation of aromatic compounds by bacteria is often initiated by dioxygenases, which catalyze the enantiospecific addition of dioxygen to the aromatic ring to form cis-dihydrodiols. Bacterial three-component dioxygenases consist of reductase and ferredoxin components which transfer electrons from NAD(P)H to a terminal oxygenase. This oxygenase, an iron-sulfur protein (ISP), catalyzes the incorporation of oxygen into the substrate and determines substrate specificity. In most cases, ISP consists of two different subunits. The large subunit contains a Rieske [2Fe-2S] center and non-heme mononuclear iron. The role of the small subunit is not clear. To assess the contributions of the large and small subunits of ISP to substrate specificity, hybrid dioxygenase enzymes were formed by constructing hybrid operons or by coexpressing genes from two compatible plasmids in *Escherichia coli*. A hybrid 2-nitrotoluene dioxygenase operon containing the C-terminal coding region of the ISP large subunit gene from the 2,4-dinitrotoluene dioxygenase system was constructed. The resulting enzyme had an increased substrate range and altered regioselectivity and enantioselectivity, although product formation rates were extremely low. Substitution of the small subunit gene from 2,4-dinitrotoluene dioxygenase into this hybrid operon resulted in an enzyme unchanged in substrate specificity but with increased product formation rates. Activities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenases containing four different ISP small subunits were also analyzed. In the active hybrids, replacement of small subunits affected the rate of product formation but had no effect on the substrate specificity, regioselectivity, or enantioselectivity of the enzyme with the substrates tested. These studies indicate that the C-terminus of the ISP large subunit controls substrate range, regioselectivity, and enantioselectivity. The small subunit of the terminal oxygenase component is essential for enzyme activity and appears to interact with C-terminus of the ISP large subunit. The small subunit does not appear to play a role in determining substrate specificity in these enzymes.

XXXXVI

Many Pseudomonads can grow on aliphatic and aromatic hydrocarbons that occur in the environment. For this they use pathways in which mono- and dioxygenases first oxidize these compounds with molecular oxygen to form alcohols or epoxides. These oxygenases generally have a wide substrate range, are regiospecific and (in the case of epoxides) stereospecific, and can usually oxidize a dozen or more substrates, something that is quite difficult to do with conventional chemistry. Hence the biotechnological potential of such enzyme systems.

Oxygenases often consist of several components, one of which (the oxygenase proper) is generally a membrane protein. In addition, monooxygenases require cofactors (NADH, NADPH) for the reduction of the oxygen atom which is not incorporated into the substrate. As a result, monooxygenase catalyzed reactions are carried out *in vivo*, in whole cell systems, where these complex enzyme systems are expected to perform optimally, and cofactor regeneration occurs through normal metabolism.

Monooxygenase substrates are typically apolar, non-water soluble compounds, such as alkanes or benzene derivatives. They are therefore supplied to growing cells either in limiting amounts, in the vapor phase or as a bulk liquid phase. We have explored the latter case, and have studied the characteristics of the resulting two-liquid phase systems in the past decade, using batch, fed-batch and continuous cultures.

We have maximized cell growth in such media, reaching cell densities of 40 g/l (dry mass) for *E. coli* recombinants and 100 g/l (dry mass) for *Pseudomonas* strains. In continuous cultures, cell densities between 3 - 20 g/l (dry mass) can be maintained. The above processes are illustrated by our work on the production of chiral styrene epoxides, chiral epoxyalkanes, terminal alkanols and alkanolic acids. Down stream processing of such products from the separated phases is now being developed. For some of these bioconversions, productivities equivalent to 3-5 tons/m³•yr have been attained. These productivities are beginning to be attractive from an industrial point of view, and several industries have in fact begun exploring biological oxidations for the production of chemicals.

Thus, these systems have now matured to the point where there is not much difference in the attainable growth rates and the maximum cell densities which can be achieved with *Pseudomonas* strains or *E. coli* recombinants in two-liquid phase systems containing 10% to 50% (v/v) apolar phase, as compared to strictly aqueous media. It is to be expected that bioconversion rates of apolar compounds in two-liquid phase systems can likewise be carried out at rates similar to those seen in aqueous systems.

There are several questions which we are now trying to answer. What are the maximum rates at which apolar compounds can be transferred from apolar phase droplets in the aqueous phase to cells growing in this aqueous phase, i.e., what are the maximum space-time yields attainable in two-liquid phase fermentations under practical conditions? To what extent can oxygen enriched air be used for aeration? What safety regimes should be considered in working with flammable organic solvents?

In addition, we have carried out detailed cost analyses, incorporating data for growth and bioconversion rates and for the down stream processing necessary to attain desired product purity. Based on these analyses it is possible to determine how optimal product formation and purity might be reached and how further research should be focused to reduce the overall cost of these biocatalytic processes. This information can be used to help determine the economic feasibility of a particular process.

XXXXVII

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Product recovery from dilute aqueous solutions is a major problem in biotechnological processes based on whole cells. This statement holds especially if compounds are produced which are inherently toxic to living cells. An important group of such products are low molecular weight compounds which have a hydrophobicity near to the hydrophobicity of bacterial membranes. Such compounds partition from the aqueous phase to the membrane, resulting in loss of integrity of this vital component.

In 1989, Inoue and Hirokoshi (1) reported the isolation of a toluene resistant *Pseudomonas putida* strain able to grow in a two-phase toluene-water system. This finding came as a surprise because toluene is extremely toxic for living organisms (2,3). It accumulates in and disrupts membranes. The observations of Inoue and Hirokoshi have been confirmed by several research groups. The solvent-resistant bacteria allow a new degree of freedom in producing by whole cells of toxic fine chemicals such as catechols, medium chain alcohols and epoxides.

The presentation will focus on mechanisms by which *Pseudomonas putida* S12 is able to overcome the toxicity problems of toluene and other organic solvents. Key elements to be discussed are adaptations at the membrane level such as the formation of trans-unsaturated fatty acids (4), and active efflux of solvents from the membrane (5).

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XXXXVIII

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An aspect of biodegradation that has received little attention is how bacteria sense and acquire aromatic compounds from the environment. *Pseudomonas putida* is chemotactically attracted to a variety of aromatic acids, including 4-hydroxybenzoate (4-HBA), 3-chlorobenzoate and toluates. Recently, modified chemotaxis assays were developed to examine if aromatic hydrocarbons, which tend to be less soluble and more volatile than aromatic acids, are also chemoattractants. Two naphthalene-utilizing strains of *Pseudomonas* were found to be strongly attracted to naphthalene and this capability was induced by growth on either naphthalene or salicylate.

Analyses of mutants that were nonchemotactic to 4-HBA led to the identification of PcaK, a hydrophobic protein with twelve membrane spanning regions that is similar in sequence to transport proteins, and not to known chemotaxis proteins. However, PcaK is required for chemotaxis to 4-HBA, benzoate and nonmetabolizable aromatic acids, and thus appears to function as a chemoreceptor as well as a transporter. The PcaK protein, when expressed in *E. coli*, catalyzed the intracellular accumulation of 4-HBA against a concentration gradient, confirming its permease function. A distinct aromatic acid permease family has recently been defined within the major facilitator superfamily of transport proteins that includes PcaK, as well as TtdK, proposed to function in transport of 2,4-dichlorophenoxyacetate, and BenK, proposed to function in benzoate transport.

Work to elucidate the unusual role of PcaK in chemotaxis has focused on using site-directed mutagenesis to examine the relationship between the transport and chemotaxis functions of PcaK. Also, general chemotaxis genes of *P. putida* were cloned and sequenced. The results indicate that *P. putida* uses a sensory signaling pathway for chemotaxis that is similar to the well-studied *E. coli* pathway. It is likely that sensory information from PcaK feeds into the general chemotaxis pathway either directly or via one or a few "coupling" proteins. The basis for naphthalene chemotaxis is less clear. However, NAH-plasmid cured strains that could grow on naphthalene by means of cloned naphthalene-degradation genes, were not chemotactic to naphthalene. This indicates that the NAH plasmid harbors genes that are specifically required for naphthalene chemotaxis.

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XXXXIX

Polyhydroxyalkanoate biosynthesis in *pseudomonades* and related bacteria: molecular analysis of the genes and utilization for production of polyesters in recombinant bacteria

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Polyhydroxyalkanoic acids (PHA) represent a complex class of storage compounds for carbon and energy occurring as insoluble cytoplasmic inclusions in a wide range of different bacteria. They were also detected in many pseudomonades and related Gram-negative bacteria. PHA synthases, which catalyze the key step in PHA biosynthesis, have been cloned and analyzed at a molecular level from more than 20 different bacteria. In addition, genes for enzymes catalyzing the conversion of intermediates of central or otherwise important pathways into hydroxyacyl-Coenzyme A thioesters, which represent the substrates for PHA synthases, have also been cloned. This contribution summarizes the recent knowledge on the PHA synthases in *Pseudomonas* sp., *Alcaligenes eutrophus* and related bacteria. It will be shown that these genes can heterologously be expressed in functional active forms in other bacteria conferring the capability to synthesize and accumulate PHA. Examples will be the synthesis of poly(4-hydroxybutyric acid) homopolymer in *E. coli*, the synthesis of PHA consisting of medium-chain-length 3-hydroxyalkanoic acids in *E. coli* and the synthesis of copolymers consisting of 4-hydroxyvaleric acid in PHA-negative mutants of *Pseudomonas putida* and *Alcaligenes eutrophus*. The impacts of these findings with respect to the biotechnological production of PHA with recombinant bacteria or even higher organisms will be discussed.

XL

Pseudomonas aeruginosa Outer Membrane Proteins: Structure/function Relationships

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Gram negative bacteria, like *Pseudomonas aeruginosa* have outer membranes which serve as size dependent permeability barriers. This property is created by the presence of a class of proteins termed porins which form water-filled diffusion channels across the outer membrane. There are three classes of porins. The general porins, permit relatively non-specific passage of hydrophilic molecules (e.g., β -lactam antibiotics, sugars, etc.), providing these molecules are smaller than the exclusion limit of the porin channel. In the case of *P. aeruginosa* the most predominant non-specific porin, OprF, has a large exclusion limit but functions quite inefficiently. This contributes to the high intrinsic antibiotic resistance of this organism. At the same time, low outer membrane permeability makes it important for *P. aeruginosa* to have effective alternate systems for passage of substrates present in low levels in the environment. Such alternate systems utilize substrate-specific porins with a defined binding site within the channel, or gated channels that are presumably only functional in the presence of a specific substrate. Interestingly, all of the above channel types have thematically similar structures, that utilize a variety of genetic methods, including linker and epitope insertion mutagenesis, PCR-directed site specific mutagenesis, and site directed mutagenesis, combined with model membrane studies, to decipher structure-function relationships in porin proteins. This talk will discuss the basic molecular differences between non-specific and substrate specific porins, with particular emphasis on protein OprD, a basic amino acid and imipenem antibiotic specific porin, and OprP, a phosphate specific porin. The data indicate that these substrate specific porins, in addition to having amino acids which provide specific binding sites, contain constricted channel entrances which can strongly influence the specific passage of substrates through these channels.

XLI

Pumps, pumps, and more pumps: efflux-mediated multidrug resistance in *Pseudomonas aeruginosa*

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Intrinsic antibiotic resistance in *P. aeruginosa* results from a synergy between low outer membrane permeability and the operation of broadly specific drug efflux pumps. The *mexA-mexB-oprM*-encoded system forms a tripartite pump comprised of a cytoplasmic membrane, proton-motive force-dependent exporter (MexB), an outer membrane porin-like protein (OprM) and a so-called membrane fusion protein (MexA) which links the two. Expressed constitutively, thereby contributing to intrinsic resistance in wildtype cells, the operon is hyperexpressed in *nalB* mutants (1), leading to the elevated multidrug resistance of these mutants (2). Homologous efflux systems, encoded by the *mexC-mexD-oprJ3* and *mexE-mexF-oprN4* operons, are not expressed constitutively but are expressed in *nfxB* and *nfxC* multidrug resistant mutants, respectively. Each system displays a unique profile of substrate antibiotics. MexAB-OprM displays the greatest capacity for antibiotic substrates, exporting macrolides, quinolones, novobiocin, tetracycline, chloramphenicol, trimethoprim, sulphonamides, fosfomycin, β -lactamase inhibitors and β -lactams. MexCD-OprJ accommodates many of the above with the exception of most β -lactams. MexEF-OprN exports the most limited range of substrates, effluxing quinolones, chloramphenicol and trimethoprim. It is likely that these differences reflect the natural function of each system in exporting a distinct physiological substrate(s) which has yet to be identified. Via the construction of strains expressing hybrid efflux systems (i.e. MexAB-OprJ, MexCD-OprM), we have shown that the cytoplasmic membrane-associated components are the determinants of substrate specificity. The operation of the MexAB-OprM efflux system is at least partially dependent upon the cytoplasmic membrane-associated energy coupling protein TonB previously implicated in the operation of outer membrane gated channels involved in the uptake of iron-siderophore complexes (5). This suggests that OprM (and OprJ/OprN) may operate as energy-dependent gated channels which open in response to antibiotics in the export pathway. The regulation of *mexAB-oprM* is complex, involving MexR, a homologue of the *Escherichia coli* multiple antibiotic resistance (*mar*) repressor MarR, which represses *mexAB-oprM* in wildtype cells but is required for overexpression of the efflux genes in *nalB* mutants (1). The operon is growth phase-regulated, with expression decreasing in lag phase and increasing to a maximum in mid-to-late log phase. This growth phase regulation is maintained in *mexR* null mutants and in *nalB* strains but is lost in mutants defective in LasR, the quorum sensing regulator (6). Thus, expression of *mexAB-oprM* appears to be cell density-dependent. The nature of the involvement of *mexR* and *nalB* in the regulation of *mexAB-oprM* expression remains, however, to be elucidated.

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XLII

The essential *Pseudomonas asd* gene: Its use as a selectable marker and derivation of attenuated mutants.

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We recently cloned and characterized the *Pseudomonas aeruginosa asd* gene and described some of the properties of its gene product, aspartate- β -semialdehyde dehydrogenase (Asd). Asd is required for the biosynthesis of lysine, methionine, threonine, and diaminopimelic acid (DAP). In Gram-negative bacteria, DAP is essential for proper crosslinking of the peptidoglycan and in the absence of DAP, *asd* mutants lyse rapidly. Since mammalian tissues do not produce DAP, *Salmonella* and *Shigella* attenuated *asd* mutants have been exploited for construction of balanced, lethal vaccine delivery systems. To date, in *Pseudomonas* sp. only the *purA* marker has been exploited for construction of an attenuated *P. aeruginosa* mutant. In the present studies, we (i) further exploited the previously characterized *asd* gene for construction of a *P. aeruginosa* attenuated mutant; (ii) explored the use of *asd* as selectable marker; and (iii) demonstrated the use of *asd*-specific primers for cloning and characterization of the *Burkholderia pseudomallei asd* gene.

Utilizing a previously described gene replacement strategy, the *P. aeruginosa* chromosomal *asd* gene was deleted. As expected, the mutant was auxotrophic for lysine, methionine and threonine, and required DAP for growth, even on rich media. In the rat lung agar bead chronic infection model, the *asd* mutant was completely attenuated and no viable bacteria could be recovered after 1, 3 and 7 days, respectively. In contrast, utilizing the same initial inoculum, wild-type PAO1 survived normally and viable cells could be recovered in large numbers over the same time period. These results clearly demonstrated that PAO *asd* mutants are completely attenuated and thus could possibly be developed into a live attenuated vaccine.

To facilitate use of cloned *asd* as a selectable marker, *P. aeruginosa asd* was genetically engineered to remove unwanted internal restriction sites and to create a unique *NdeI* site immediately upstream of the putative ribosome-binding site. The result was a promoterless *asd* cassette that can easily be excised from its parental vector by virtue of several unique flanking restriction sites. When *asd* was cloned into a broad-host-range vector in place of the resident *bla* gene, the plasmid was stably maintained for 100 generations in a PAO1 Δ *asd* mutant in the absence of DAP but was rapidly lost from DAP-supplemented cultures. Moreover, to demonstrate the use of *asd* in routine cloning experiments, the popular pUC18/19 cloning vectors were modified such that their *bla* gene was replaced with the PAO *asd* gene. In conjunction with a newly constructed, highly transformable, and high-quality plasmid DNA producing *E. coli* Δ *asd* host strain, the new *asd*-based vectors offer some unique advantages. These include (i) elimination of the need for phenotypic expression prior to plating of electroporation/transformation mixtures and (ii) no "cross-feeding", i.e., no satellite colonies and all cells in a culture actually contain plasmids. Similar broad-host-range cloning vectors have also been developed and in combination with defined *Asd* strains will be useful for studies involving recombinant plasmids in situations where antibiotic selection for stable plasmid maintenance is not feasible, e.g., *in vivo* models and biofilms.

To demonstrate the usefulness of previously developed PCR primers for cloning of the *asd* genes from other pseudomonads, or *Pseudomonas*-like species, a partial *asd* coding sequence was amplified from *B. pseudomallei* genomic DNA, and the complete gene was cloned and sequenced. An *asd* deletion in this organism is currently being constructed and will serve several purposes. First, an attenuated, biosafe *B. pseudomallei* strain will facilitate laboratory studies with this organism, which in the United States currently must be handled in a level 3 facility. Second, with proper development an attenuated mutant could serve as the basis for a live vaccine strain.

XLIII

Cell envelope proteins and rhizosphere colonization of *Pseudomonas fluorescens*

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The outer membrane protein OprF constitutes the most abundant cellular protein in *Pseudomonas fluorescens* cells. The highly conserved C-terminal part of this protein displays homology with various peptidoglycan-binding proteins, ranging from OmpA to MotB (1).

Analysis of OprF proteins from rhizosphere isolates of *P. fluorescens* revealed the occurrence of two types of OprF proteins, differing by the length and amino acid sequence of their central domain. Strains like F113 and WCS365 have a cysteine-containing domain, as found in OprF proteins from *Pseudomonas aeruginosa* and *Pseudomonas syringae* and in the outer membrane protein CD from *Branhamella catarrhalis* (2). In other strains like OE 28.3, MF0, and NRRL B-15132, the equivalent region consists of a proline-rich repeat (3). Both types of OprF are also found among other fluorescent *Pseudomonas* species.

When incorporated in planar lipid bilayers, OprF proteins from strains MF0 and OE 28.3 display similar low single-channel conductance values (4). These values are reduced threefold when using OprF extracted from these strains grown at low temperature (5).

OprF-negative mutants are impaired in their ability to colonize plant roots either in a gnotobiotic system (OE 28.3/tomato) or in soil microcosm (F113/sugarbeet).

A gene encoding a putative novel sigma factor of the ECF family is present upstream of the *oprF* gene. Insertional inactivation of this gene results in a reduced amount of OprF in *P. fluorescens* OE 28.3.

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XLIV

The resistance of *Pseudomonas aeruginosa*: Search for its molecular basis

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Pseudomonas aeruginosa is notorious for its intrinsic resistance to many antibiotics. Following the discovery of OprF as its major porin (1), this protein was found to produce a level of permeability about 40 times lower than do the classical porins of *Escherichia coli* (2), and this seemed to explain the exceptionally low permeability of the *P. aeruginosa* outer membrane (3, 4). Nevertheless, the apparent discrepancy between the large pore size (1) and the low permeation rate has produced much skepticism. One particular investigator believed in a simplistic hypothesis that the pores must be small to produce low levels of permeability, and published a series of papers based largely on misinterpretation of his own data to fit this preconceived notion. Although this controversy created much confusion, the original observation of large pore with low permeability is now largely accepted (5). We have tried to find the correct explanation for this apparent discrepancy. Study of the OprF homolog of *E. coli*, OmpA, showed that it can also produce pores of normal size at very low efficiency (6), and fractionation of OmpA population based on its pore-forming activity led to the demonstration that it consists of two components, the majority (>90%) totally incapable of forming open channels, and the minority (2-3%) capable of forming channels at normal efficiency (7). Most recently, we succeeded in carrying out this fractionation on the basis of size. Thus with both OmpA and OprF, apparently oligomeric forms that are eluted earlier upon gel filtration have a much higher pore-forming activity than the majority population, which is monomeric and cannot produce channels. This then confirms the earlier prediction that only a minor fraction of OprF produces channels. Additionally, insight from another direction into the mechanism of drug resistance was obtained by the discovery of multidrug efflux pumps (8, 9), and we are now close to at least the first approximation of the complete explanation of the intrinsic resistance phenotype of *P. aeruginosa*.

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XLV

POSTERS ABSTRACTS
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9.00 am-8.00 pm

The *Pseudomonas aeruginosa* *gluM* gene, which encodes a putative inner membrane carbohydrate transport ATP-binding protein, is closely linked to the glucose transport OprB porin gene.

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The inducible binding protein-mediated glucose transport system in *P. aeruginosa*, which is required for efficient glucose utilization in glucose-limited minimal medium, requires the periplasmic glucose-binding protein (GBP) (1), the carbohydrate-selective outer membrane OprB porin (2, 3) and a two-component response regulator, GltR (4). Using λ TnPhoA mutagenesis of a genomic clone containing the cloned *oprB* gene (5) and flanking regions, an open reading frame, designated *gluM*, was found upstream of the *oprB* gene. This ORF encodes a putative polypeptide of approximately 35kDa with the stop codon located 500 bp upstream of the start of translation of the *oprB* gene. Sequence analysis of the predicted translation product revealed strong homology (54-65% identity) to several inner membrane carbohydrate transport ATP-binding proteins, including the *E. coli* MalK, the maltose/maltodextrin-transport protein; *S. mutans* MsmK, the multiple sugar-binding transport protein; *A. radiobacter* Lack, the lactose transport protein, and *E. coli* UgpC, the sn-glycerol-3-phosphate transport ATP-binding protein. Weaker homology (~25% identity) was found with the *P. aeruginosa* Braf, the high-affinity branched-chain amino acid transport ATP-binding protein. A 600 bp fragment from the C-terminal region of *gluM* also hybridized to *EcoRI*-digested genomic DNA from *P. fluorescens*, *P. putida* and *P. chlororaphis*. The cloned *gluM* gene was mutagenized in vitro by deletion of the C-terminal half of the gene. This construct was cloned into pEX100T gene replacement vector (6) and then mobilized into *P. aeruginosa* H103 to replace the wild type gene by homologous recombination. We are currently analyzing the effect of this deletion on the transport of glucose via the high-affinity system in the *gluM* deletion mutant strain in order to evaluate the role of GluM as the inner membrane component of the glucose ABC transporter in *P. aeruginosa*.

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Molecular and expression analysis of the *pur* gene encoding the polyester polyurethane degrading enzyme from *Comamonas acidovorans* strain TB-35

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We isolated a bacterium, *Comamonas* (formerly *Pseudomonas*) *acidovorans* strain TB-35, which could utilize solid polyester polyurethane (PUR) as the sole carbon and nitrogen sources and found that the esterase released from this strain played a major role in the PUR degradation (1, 2). Furthermore, we purified the PUR degrading enzyme, PUR esterase, from strain TB-35. N-terminal amino acid sequence of the PUR esterase was identified and an oligonucleotide probe was synthesized on the basis of this sequence. A 10-kb *EcoRI* fragment of the total DNA of strain TB-35 hybridized with the DNA probe was constructed in the pUC19 and transformed into *E. coli* DH10B cells. Deletion analysis of the fragment identified a 3.0-kb fragment with the esterase activity and sequence analysis revealed an open reading frame (ORF) of 1647 bp that was located downstream from a potential ribosome-binding site. The ORF encoded a polypeptide of 548 amino acid residues, and the deduced molecular mass of this polypeptide was 55kDa. The amino acid sequence included the regions for the predicted catalytic domain of esterase (-G-X-S-X-G-) and for the putative hydrophobic PUR binding domain. This ORF might be *pur* gene encoding PUR esterase, since the deletions of this ORF led to complete loss of the esterase activity. The PUR esterase expressed in *E. coli* was purified. The purified PUR esterase degraded the PUR, and the components of the polyester segment of PUR, adipic acid and diethylene glycol, was detected as the products. These results indicated that the cloned gene product hydrolyzed the ester-bond of the PUR, and so the ORF (*pur*) was coding for the PUR esterase.

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Emergence and selection of antibiotic multiresistant mutants is one of the most important problems for the treatment of hospital infections by opportunistic pathogens. The phenotype of multiresistance is the consequence of expression of cryptic efflux-pump systems present in wild-type strains, so that multiresistant strains can emerge by a single mutation step. It has been proposed that, at least for some experimental models, bacteria can present increased mutagenesis under selection conditions (adaptive mutation). In the present work, we have explored whether or not this situation can apply for the emergence of antibiotic multiresistant mutants. For this purpose, *Pseudomonas aeruginosa* PAO1 was subjected to selection with tetracycline in solid LB medium, and the emergence of resistant colonies over time was studied. The frequency of mutation raised from 3×10^{-6} on day two after plating to 1.3×10^{-4} on day six after plating. Colonies from different days were tested for antibiotic resistance and the presence of outer membrane proteins (OMP) diagnostic of multiresistance. All mutants presented an antibiotic multiresistant phenotype, and the presence of a new OMP with a molecular size of 50 kDa. was detected in all of them. This phenotype was stable for at least 20 generations, indicating that the phenotype is the consequence of a mutation event, and not to the induction of the pump(s). It has been described that *Pseudomonas aeruginosa* possesses at least three multidrug resistance (MDR) systems. We have studied the variability of the MDR mutants emerging during tetracycline selection. At least four different morphotypes were detected. This result shows that a single clone of *P. aeruginosa* can present a high populational variability. The relevance of these results for the populational dynamics of multidrug resistant bacteria will be discussed.

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Clinical isolates of *S. maltophilia* are often intrinsically resistant to several antibiotics commonly used in clinical practice, therefore we tested for the presence of a multiple antibiotic resistance (MDR) system in this species. The clinical isolate *S. maltophilia* D457 was plated on LB containing tetracycline at a concentration twice its MIC. Resistant mutants were selected at a frequency of 1.9×10^{-6} . MICs of several antibiotics were determined for one of the mutant (D457R) and results showed that the resistant strain was less susceptible not only to tetracycline but also to quinolones and to chloramphenicol. This was not observed for aminoglycosides or β -lactam antibiotics. Transport assays of tetracycline and norfloxacin indicated that intracellular accumulation was smaller in the resistant strain as compared to the wild type. However, accumulation of both antibiotics increased significantly in the presence of a proton-motive force uncoupler. The multiple antibiotic resistance phenotype was linked to the expression of an outer membrane protein of 54 kDa both in the resistant strain obtained in the laboratory and in clinical strains that presented intrinsic multiple resistance. These results indicate that multiple antibiotic resistance phenotype in *S. maltophilia* is due to the presence of at least one efflux pump system as described for other Gram-negative bacteria. Colonies of the resistant strain D457R were smaller than those of their isogenic wild-type one D457. When analyzed by electron microscopy, D457R cells were also smaller than their D457 counterpart. Co-culture experiments demonstrated that acquisition of a multidrug resistance phenotype produces a decrease in the fitness of bacteria. The ecological significance of these results will be discussed.

Phenanthrene degraders from the group of fluorescent pseudomonads isolated from the rhizosphere

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In this study we isolated phenanthrene degraders belonging to the group of fluorescent pseudomonads by combining part of the selective force of two former described media. The Gould's S1 medium is selective for fluorescent pseudomonads especially due to two compounds, Sodium Lauryl Sarcosine, SLS and the antibiotic Trimethoprim, Tp (Gould *et al.* 1985). The spray plate technique has been developed to isolate degraders of certain hydrophobic compounds not soluble in ordinary agar media (Kiyohara *et al.* 1982). The hydrophobic compound is sprayed on top of a minimal agar medium, leaving an opaque layer. Bacteria capable to degrade the compound will appear as a small colony with a surrounding clearing zone. By adding the two compounds, SLS and Tp, from the Gould's S1 medium to the minimal agar medium, it was possible directly to isolate fluorescent pseudomonads from the rhizosphere, capable of producing clearing zones, without any first isolation of pseudomonads with subsequent screening of degraders or vice versa. Enumeration and isolation of fluorescent pseudomonads showed clearly difference between two types of soil. Only phenanthrene degraders (from the fluorescent group) showed up in a soil from a coal gasification site whereas an agricultural organic soil did not contain fluorescent pseudomonads degrading phenanthrene. We isolated 23 phenanthrene degraders producing clearing zones from the rhizosphere of barley roots. All of these 23 isolates showed to be member of the RNA homology group 1 of the pseudomonads according to API test system and classic taxonomic tests. Beside this, 41 colonies not producing clearing zones were isolated to make a more specific characterisation of the bacteria appearing on the selective agar medium. API test system showed that 24 of these 41 isolates were determined to be member of the genus *Pseudomonas* and 17 of these produced fluorescent colonies in UV-light. The degradation of phenanthrene by the 23 degraders was followed in a hydrocarbon minimal medium both by visual inspection (development of colour) and by monitoring the disappearance of phenanthrene by gas chromatography. There was accordance between the development of colour in the medium and the disappearance of phenanthrene, however the degradation products were not determined.

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Anthranilate restricts the growth of the root pathogen *Pythium* and protects plants against *Pythium*-induced damping-off

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Pseudomonas aeruginosa PNA1, isolated from chickpea roots in India, inhibits mycelial growth of several root pathogens (*Fusarium oxysporum*, *Fusarium udum*, *Rhizoctonia bataticola*, *Sclerotium rolfsii*, and *Pythium debaryanum*) in culture. In Glucose-casaminoacid-yeast extract (GCY) medium, PNA1 produced two phenazine compounds, phenazine-1-carboxylic acid (PCA) in large amounts, and oxychlorophane (OCP) in minor amounts. Two classes of Tn5 mutants with reduced capacity to inhibit *Fusarium* growth were analysed: prototrophs deficient for phenazine production, and tryptophan auxotrophs. One such tryptophan auxotroph, which had the Tn5 inserted in the *trpC* gene, did not produce phenazines in GCY medium, and excreted anthranilate in the medium. This mutant retained however an antifungal activity against *Pythium* while the phenazine-negative mutants had lost completely their antagonism against all fungi tested. Pure anthranilate was also found to restrict the growth of *Pythium in vitro* and to protect tomato and salad seedlings from *Pythium*-induced damping-off.

Adaptation of *Comamonas testosteroni* TA441 to phenol by increasing the catabolic gene expression

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Comamonas testosteroni TA441 was isolated from a termite ecosystem. It took about two to three weeks for the strain to start to grow on phenol as the sole carbon and energy source. When once strain TA441 grew on phenol, it got ability to utilize phenol without the long lag period. The adaptation of strain TA441 to phenol was a reproducible phenomenon and the ability of the adapted strains to degrade phenol was stable, suggesting that some genetic changes had occurred during incubation with phenol. The adapted strain designated as strain P1 expressed high activities of phenol hydroxylase (PH) and catechol 2,3-dioxygenase (C23O) in response to phenol indicating that phenol was degraded via *meta*-cleavage pathway. The genes for phenol degradation were cloned from both strains TA441 and P1. Sequence analyses revealed that the structural genes encoding multi-component PH and C23O (*aphKLMNOPQB*), and the gene for a putative transcriptional regulator of NtrC family (*aphR*) were located in a divergent transcriptional organization like in the case of the *dmp* operon for (methyl)phenol catabolism in *Pseudomonas* sp. strain CF600. No difference was found between TA441 and P1 in the sequences of the *aphR* gene and of the intergenic promoter region between *aphK* and *aphR*. However, transcriptional activities from the *aphK* and *aphR* promoters probed by *lacZ* fusions were significantly higher in strain P1 than in strain TA441.

Cloning of the *Pseudomonas stutzeri* OX1 genes involved in the *o*-xylene and toluene meta pathway and in the regulation of the upper pathway.

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Pseudomonas stutzeri OX1 degrades toluene and *o*-xylene through two consecutive monooxygenations of the aromatic ring leading to the formations of mono- and dimethyl-phenols and catechols respectively. The degradation proceeds through the extra diol cleavage of the methylated catechols catalized by a catechol 2,3-dioxygenase (C2,3O). The genes coding for the toluene/*o*-xylene monooxygenase (ToMO) are chromosomally located in a 6 kb *NotI*-*DraI* fragment (1). At approx. 7 kb distance from the *DraI* site we identified a region coding for a phenol hydroxylase, a catechol 2,3-dioxygenase, a hydroxymuconic semialdehyde hydrolase, and a hydroxymuconic semialdehyde dehydrogenase activity. Phenol hydroxylase displays a broad range of substrates; the minimal DNA region required to detect activity is 4.8 kb long and shows homology with the genes coding for the subunits of the *Pseudomonas* sp. CF600 phenol hydroxylase. Phenol hydroxylase and C2,3O genes are divergently transcribed with respect to the ToMO ones, suggesting that the toluene/*o*-xylene catabolic pathway may be organized in two operons.

In *P. putida* PaW340 cells carrying a recombinant plasmid which includes the ToMO genes and the flanking regions the ToMO activity was found to be inducible by both the hydrocarbons and their phenolic intermediates. A *trans* acting element which positively affected the ToMO expression was mapped immediately downstream from the *NotI* site which represents the end of the ToMO gene cluster; we are now delimiting and sequencing this region. A *cis* acting regulatory region was tentatively mapped at least 1 kb upstream from the *DraI* site. To study the regulatory elements a reporter system was constructed by cloning the *P. stutzeri* C2,3O gene under the putative ToMO promoter; we are now assaying the inducibility of C2,3O activity in *P. putida* PaW340 cells carrying the DNA region where the *trans* acting element was mapped.

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The dioxin dioxygenase is the key enzyme in the dibenzo-*p*-dioxin and dibenzofuran degradative pathways of *Sphingomonas* sp. RW1. Improvement of its catalytic properties in terms of the range of chlorinated substrates requires a genetic characterization of this Class IIA dioxygenase. Considerable effort has been invested to clone and characterize the genes of this three-component dioxygenase, but until now was unsuccessful. Finally, with the purification of the corresponding proteins (1) and the availability in the databases of numerous degradative enzyme sequences, a PCR-based strategy using degenerate primers designed with consensus and N-terminus sequences was developed. Specific probes for the structural genes specifying the dioxin dioxygenase and its electron supply system were obtained and used to screen a genomic library. The gene encoding the ferredoxin, *fdx1*, was identified and shown to not be linked to the dioxin dioxygenase genes but rather clustered with genes encoding two putative decarboxylases and an atypical glutathione-S-transferase (2). *Fdx1* has been shown to contain a putidaredoxin-type [2Fe-2S] cluster and exhibits redox properties similar to those of putidaredoxin, a ferredoxin associated with a three-component monooxygenase. We have also cloned and sequenced the structural gene encoding the reductase component A2 (*redA2*). Investigation of the biochemical properties of this protein shows marked similarities with Class I cytochrome P₄₅₀ reductases (3). We have also cloned the genes *dxnA1* and *dxnA2* encoding the initial dioxin dioxygenase. The sequence of the α and β subunits of the dioxin dioxygenase exhibits only weak similarities with other three component dioxygenases but some motifs such as an Fe(II)-binding site or [2Fe-2S] cluster ligands are well conserved. No genes encoding the electron supply system were identified in the close vicinity of the dioxygenase genes, revealing a gene organization different from that of most of three-component dioxygenases. Expression of the cloned genes was carried out in *Escherichia coli* and the activity of the dioxin dioxygenase measured in resting cell assays. The dioxin dioxygenase was fully active towards dibenzofuran, dibenzo-*p*-dioxin and biphenyl when coexpressed with *fdx1* and *redA2* cloned from *Sphingomonas* sp. RW1 but inactive when coexpressed with a Class IIB electron supply system (a Rieske-type [2Fe-2S] ferredoxin and its specific reductase). The dioxin dioxygenase attacks dibenzo-*p*-dioxin and dibenzofuran at a bridge position whereas all other dioxygenases, such as biphenyl dioxygenases, hydroxylating multi-ring compounds almost always preferentially attack in neighbouring carbon atoms not involved in bridges between rings. The fact that the genes encoding the different components of the dioxin dioxygenase are unlinked in RW1 explains why usual cloning strategies were unsuccessful.

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Identification of *Pseudomonas syringae* pv. *pusi* by PCR amplification with specific oligonucleotide primers: evidence for phylogenetic divergence among races.

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We have used random amplified polymorphic DNA (RAPD)-PCR fragments from *P. syringae* pv. *pusi* as a basis for the design of specific oligonucleotide primers. Fragments from isolates 1691 (race 7) and 203 (race 2), were cloned in pUC18 and the resulting sequences were used to design two pairs of oligonucleotide primers. All four primers were used in combination with DNA amplification reactions among a range of isolates of *P. syringae* associated with pea. All strains of *P. syringae* pv. *pusi* produced one of two PCR bands, either a 272 bp or a 132 bp fragment, while bands were not detected in other closely related *P. syringae* pathovars. From these results we propose that isolates of *P. syringae* pv. *pusi* can be classified into two phylogenetic groups: those producing a 272 bp fragment with the specific primers were designated group I, while those producing a 132 bp fragment were designated group II. All strains examined in races 1, 5 and 7 were in group I, and all those in races 2 and 6 were in group II. However races 3 and 4 comprised strains from both phylogenetic groups.

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Pseudomonads are a major component of the microflora colonizing the surfaces of plants such as sugar-beet [1]. The population dynamics of bacteriophages infecting these bacteria are currently being investigated as part of an ongoing in situ assessment of the potential for phage-mediated gene-transfer in this environment. A crop of field-grown sugar-beet (*Beta vulgaris* var. *Amethyst*) was regularly sampled over an eleven month period after sowing. On each occasion, root and leaf samples of individual plants were examined for the presence of phage capable of infecting at least one of three strains of phytosphere bacteria isolated from the same site. Phages for all three strains were detected and classified according to their *EcoRI* restriction digest patterns, plaque morphology, and host superimmunity. It was noted that whilst phages for these strains were regularly isolated from the sugar-beet rhizosphere, a distinct temporal succession in phage population structure occurred over the growing season. In particular, a phage (with a long latent period and large burst size) for one of the strains, was seen to dominate samples early in the season, whilst another phage (with a short latent period, small burst size and unique multi-ringed plaque morphology) for the same strain, predominated later in the season. The significance of this previously unreported temporal fluctuation, in light of its potential impact on gene-transfer, is considered.

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The aerobic catabolism of aromatic compounds by microorganisms involves the initial action of dioxygenase enzymes. These enzymes catalyse the dihydroxylation of aromatic hydrocarbons e.g., benzene or toluene, to their respective *cis*-dihydrodiols. Two such enzymes, namely benzene dioxygenase (Bed) and toluene dioxygenase (Tod) have similar catalytic properties, structure and gene organisation. They consist of a reductase and a ferredoxin which transfer electrons from NADH to a catalytic iron-sulfur protein, consisting of two dissimilar subunits ISP α and ISP β arranged in an $\alpha 2\beta 2$ configuration. It has been demonstrated previously that the α subunit contains the catalytic center of the enzyme and the substrate recognition domain. Analysis of Bed and Tod has shown that the amino acid sequence of their α subunits differ by only 33 amino acids out of 450, however, they show clear differences in their substrate specificity. We have generated chimeric proteins in which the amino region of benzene dioxygenase a subunit (280 amino acids) was joined to the carboxy region of toluene dioxygenase a subunit (170 amino acids), producing ISP α BOD and in the same way, the amino region of toluene dioxygenase a subunit joined to the carboxy region of benzene dioxygenase a subunit producing ISP α TED. We have used these chimeric proteins to elucidate the region that has the primary influence over substrate specificity. ISP α BOD and ISP α TOD show higher enzyme activity towards toluene and ethyl benzene than benzene as substrate, in contrast to ISP α TED and ISP α BED which show higher activity towards benzene. We conclude therefore, that the domain controlling substrate specificity resides in the C terminal 170 amino acids of the protein. These results are discussed in the context of the individual amino acid that differ between the carboxy regions of the two dioxygenase α subunits.

Evidences for multiple interactions in the General Secretory Pathway of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa produces a large number of extracellular proteins with toxic or hydrolytic activities. Most of them are targeted to the surrounding medium throughout the Xcp General Secretory Pathway. At least 12 Xcp proteins are required for secretion through the outer membrane. Similar secretion systems have been identified in numerous Gram-negative bacteria and certainly constitute one of the main route to the extracellular medium.

Relationships between the Xcp components have been investigated by studying their interactions within the cell envelope. The putative ATP-binding protein XcpR is associated to the inner membrane through interaction with XcpY, an integral membrane protein having a large cytoplasmic domain. Overexpression of this domain or the full-size protein strongly interferes with secretion, indicating that XcpR-XcpY complexes require other partners in the secretion machinery. Moreover, XcpR can be chemically cross-linked *in vivo* as a high molecular weight complex in which XcpY is not detected. An interaction between XcpP, an inner membrane protein, and XcpQ, the sole Xcp protein localized in the outer membrane, is also detected by cross-linking and reveals the existence of a physical connection between both membranes. As observed in the case of XcpY, high-level expression of XcpP impairs secretion, indicating that a precise stoichiometric ratio between several components may be crucial for the functioning of the pathway. These results are consistent with the view of a multi-protein secretory complex in the envelope of Gram-negative bacteria.

Physical and genetic mapping of large, conjugative, mercury resistance plasmids indigenous to phytosphere pseudomonads.

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Previous studies on the phytosphere microflora of sugar beet have demonstrated the abundance of large, self-transmissible, narrow host range plasmids that move freely within the colonising pseudomonad community conferring mercury resistance to their hosts (Lilley & Bailey, 1997). At least five genetically distinct groups of pseudomonad-associated plasmids have been characterised. Group I, III and IV type plasmids have been shown to persist in the rhizosphere and phyllosphere of sugar beet over consecutive years (Lilley *et al.*, 1996). Plasmids pQBR11 (~300 kb), pQBR55 (~150 kb) and pQBR57 (~260 kb), representing Group I, III and IV respectively, have been chosen for further analysis. Physical maps of the three plasmids were constructed using Field Inversion Gel Electrophoresis (FIGE) and cross hybridisation with isolated fragments. Each plasmid was mapped using at least three enzymes (pQBR11 : 5 fragments with *Fse I*, 10 with *Not I* and 12 with *Spe I*, pQBR55 : 5 fragments with *Swa I*, 7 with *Sfi I* and 8 with *Xba I*, and pQBR57 : 3 fragments with *Spe I*, 5 with *Sfi I* and 6 with *Not I*). These maps will enable the genetic organisation of each plasmid to be compared, once known functional regions which are genetically unique for each plasmid, i.e. origin of replication (*oriV*, pQBR11) (Viegas *et al.*, 1997), transfer function and mercury resistance, have been isolated. The comparative analysis of the basic replicons of the three plasmids will facilitate investigations into the molecular mechanisms involved in plasmid replication and transfer regulation in the environment, improve our understanding of host plasmid associations and illustrate the possible origin and co-evolutionary strategies adopted by these genetically distinct plasmids that persist in a common habitat.

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Establishment of an *in vitro* transcription system consisting of *Pseudomonas putida* components to study the activation of σ^{54} -dependent promoters of catabolic operons

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The degradation of toluene and related aromatic compounds by *Pseudomonas putida* pWW0 is determined by four *xyl* gene operons on TOL plasmid pWW0. Two catabolic operons (upper and *meta* pathway operons, preceded by their cognate promoters Pu and Pm, respectively) contain the pathway structural genes, and two others contain the regulatory genes *xylR* and *xylS* with their promoters Pr and Ps [1]. The transcription from the promoters Pu and Ps is dependent on the σ^{54} -RNA polymerase as well as XylR activator protein which binds to recognition sequences (UAS) present in the Pu and Ps promoter upstream regions. Loop formation between upstream bound activator and the promoter-bound RNA polymerase- σ^{54} complex is required for promoter activation. In the assembly of this specific promoter geometry the histone-like proteins IHF and HU are involved [2,3].

In this project an *in vitro* transcription system will be established which only consists of components from *P. putida* in order to study the influence of histone-like proteins and environmental signals on the activation of Pu and Ps.

From *P. putida* KT2442 two genes were identified (*hupB* and *hupN*) which show high homology to the *hupB* gene encoding the β -subunit of HU from *E. coli*. The *P. putida* *hupB* was found after searching for the homolog of the *Pseudomonas aeruginosa* *hupB* whereas *hupN* was identified by Nakazawa et al. (unpublished) through complementation of *E. coli* HU mutants. *hupB* and *hupN* were overexpressed in *P. putida*. Recently the nucleotide sequences of the genes encoding the two subunits of the σ^{54} IHF (*ihfA* and *ihfB*) were determined [4]. A plasmid was constructed in order to coordinately overexpress the IHF genes in *E. coli*.

To obtain the σ^{54} -RNA polymerase the purified core enzyme from *P. putida* is to be reconstituted to the σ^{54} -holoenzyme with purified sigma 54.

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The Partitioning Proteins ParA and ParB of *Pseudomonas putida*

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It is unclear what mechanisms ensure that the bacterial genomes are partitioned in the cell prior to septation. Low copy number plasmids are lost at a lower frequency than expected if they were distributed randomly in the cell. The region responsible for such active partitioning of F and P1 encodes two *trans*-acting proteins and a *cis*-acting site, termed the centromere acting sequence. A large family of genetic loci have been identified as coding for proteins belonging to one or both of the ParA/ParB families. The ParA proteins contain an NTP binding motif belongs to a superfamily of ATPases. ParB has a helix-turn-helix DNA binding motif. Such loci are found on a variety of plasmids as well as on a number of bacterial chromosomes near the replication origin, *oriC*. These include *Bacillus subtilis*, *Caulobacter crescentus* and *P. putida*.

Like their plasmid encoded counterparts the chromosomally encoded genes play a role in partitioning. Mutants of *spoOJ* (*parB*) of *B. subtilis* cause an increase in the anucleated population in a cell culture. The proteins are involved in orientation of the chromosome so that the *oriC* region of the genome is pulled towards the poles of the cell during partitioning of the nucleoid. Protein localisation studies of the ParB of *C. crescentus* and *B. subtilis* show that it is found in foci at the *oriC* regions of the partitioning chromosomes. The *B. subtilis* genes also have a role in the regulation of sporulation.

So far it is not clear if the plasmid and chromosomally encoded ParA and ParB homologues have a related function or even whether all chromosomal homologues perform the same role. In IncP plasmids the ParA/ParB homologues perform both active partitioning and global regulation. Further detailed analysis on a number of such systems is needed to determine whether they all work in the same way or whether there are a number of different processes underpinned by these proteins. To this end we are studying the *Pseudomonas putida* *parA* and *parB* homologues found near *oriC*, *orf263* and *orf290*. We have PCR-amplified and cloned these genes followed by overproduction and purification of the proteins. We are characterising both function and structure of these proteins to determine if they have a comparable role to other members of these families.

Colonization of barley roots by rhizosphere pseudomonads: Survival of
genetically modified derivatives and characteristics of colonization-deficient
mutants

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Colonization of barley roots by *Pseudomonas* sp. 418, its Tn5-induced nitrogen non-fixing mutants, and derivative derepressed in nitrogen fixation, *Pseudomonas fluorescens* 49, its mutants non-producing fluorescent siderophore and producing it constitutively was assessed by the laboratory test and in the microplot field experiment. No significant differences were found between genetically modified and non-modified strains when tested in the laboratory, and in the density of barley seedling roots colonization by them in the field. The ability of *Pseudomonas* sp. 418 to fix nitrogen was found to determine an increase in its population in barley rhizosphere at reproductive stages of plant growth. Populations of other strains tested decreased during vegetation. Population declines of the constitutive derivatives, which had longer generation time, were more intensive. Their instability was not the major factor of this. Using the vector pSUP2021, Tn5-induced mutants of *Pseudomonas* sp. 418 unable to colonize barley roots competitively, were isolated by roots replication method. Their phenotypes were defined. Defects in active movement were most frequent. They were caused by the loss of motility and inability to produce polar flagella, and by decreased rate of chemotactic movement. In one mutant cells formed long chains, preventing them to move. The other defects found were slow attachment to root surface and alteration in the production of exopolisaccharides. A Southern blot analysis indicated that the mutants carried Tn5 in *Eco*R1 fragments of different size of 8.0 to 22.0 kb. In several mutants there were found additional insertions of Tn5 or IS50.

Effect of enhanced production of indole-3-acetic acid by the biocontrol strain
CHAO of *Pseudomonas fluorescens* on plant growth and disease suppression

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We have evaluated the possibility whether enhanced production of the phytohormone indole-3-acetic acid (IAA) in a root-colonizing pseudomonad may be beneficial to plant growth. Therefore, we have fused the IAA biosynthetic genes *iaaM* and *iaaH* from *Pseudomonas syringae* pv. *savastanoi* to a constitutive promoter and introduced this construct into the biological control agent *P. fluorescens* CHAO. The transconjugant thus obtained produced up to several hundred-fold higher amounts of IAA than did the wildtype strain CHAO. When added to autoclaved soil, the IAA overproducer was deleterious to the growth of wheat. The fresh weight of the plants was reduced by 30% and roots were severely stunted as indicated by a 5-fold reduction of their length as compared with the wildtype strain. In contrast, no such negative effects could be observed in non-autoclaved soil. Interestingly, the IAA overproducer significantly improved the growth of cucumber plants: fresh weight of roots was increased by 41% and overall plant fresh weight was 26% higher than that of plants treated with the wildtype CHAO. In disease suppression tests the IAA overproducer was not superior to the wildtype strain in controlling root rot of cucumber caused by *Pythium ultimum*. Our results indicate that in a natural soil environment enhanced IAA production by *P. fluorescens* may positively influence plant growth.

Identification and characterization of the hydrogen cyanide biosynthetic genes of *Pseudomonas fluorescens* CHA0 and analysis of their regulation

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Pseudomonas fluorescens CHA0, a root colonizing soil bacterium, protects different plants from root diseases caused by a variety of pathogenic fungi. Strain CHA0 produces and releases a battery of secondary metabolites including the biocide hydrogen cyanide (HCN) and the antibiotic 2,4-diacetylphloroglucinol. These two anti-fungal compounds have been shown to be involved in the suppression of tobacco black root rot caused by *Thielaviopsis basicola* under gnotobiotic conditions (1, 2). Three genes *hcnA*, *hcnB* and *hcnC*, encoding cyanide synthase, were identified on a genomic DNA fragment complementing HCN-deficient mutants of strain CHA0. Heterologous expression of the *hcnABC* genes under the control of the T7-promoter was necessary and sufficient for the production of HCN in *Escherichia coli*. Using the same expression system in *Pseudomonas* spp. HCN production was increased 10-fold. The deduced amino acid sequences of HcnA, HcnB and HcnC share similarities with various dehydrogenases and oxidoreductases. In addition, HcnB and HcnC each seem to contain a typical FAD/NAD-binding domain.

Under oxygen-limited conditions cyanogenesis is strongly induced. An *anr* (anaerobic regulation) mutant of strain CHA0 constructed by gene replacement did not produce HCN and translational *hcn::lacZ* fusions were not expressed in this mutant. The *hcn* promoter contains a typical ANR/FNR box in the -40 region. Deletion mutagenesis of the promoter region showed that expression decreased drastically when this box was eliminated. Thus, the expression of the *hcnABC* genes is strictly controlled by ANR at the transcriptional level.

The global activator GacA, a response regulator which belongs to a two-component signal transduction system, is required for the biosynthesis of HCN and other secondary metabolites in strain CHA0 (3). In a *gacA* mutant, *hcnABC* expression was fifty-fold lower than in the parental strain, but the residual expression was still under ANR control. Preliminary evidence suggests that GacA influences cyanide biosynthesis indirectly, through the control of another regulatory element. In conclusion, ANR appears to be the primary transcriptional activator of the *hcn* promoter whereas GacA may have modulating effects in response to environmental signals. We have evidence that some of these signals are produced by the strain CHA0 itself and that they differ from homoserine lactones, the autoinducer molecules that are common among several Gram-negative bacteria.

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Toxicity of the sulfonylurea herbicide metsulfuron methyl against fluorescent pseudomonads isolated from an agricultural soil

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Fluorescent pseudomonads were isolated from an danish agricultural soil. As a result of an continuous corn production, the herbicide atrazine has been applied to the field for about two decades. The fluorescent pseudomonads were obtained by plating dilutions from a soil-phosphate buffer mixture on Gould S1 agar. It was verified that the isolated colonies were belonging to the group of fluorescent pseudomonads by the API test system and the possibility for double isolates were eliminated by REP-PCR profiling. 77 isolates were tested for their ability to grow in Davis minimal medium in the presence of increasing concentrations of the sulfonylurea herbicide metsulfuron methyl, which has the triazin structure in common with atrazine and other triazine herbicides. A rootcolonizing strain also belonging to the group of fluorescent pseudomonads previously shown to produce siderophores and antibiotics were also tested for its ability to grow in the presence of a large scale of concentrations of metsulfuron methyl. It was shown that the herbicide had an inhibitory effect on the microbiological growth. The concentration needed to cause a reduction of the cells growth was not identical for all the strains. For some strains, the inhibitory concentration (13,11 µM) was not far exceeding the concentration we could expect to be located in the field at microenvironments as a result of application of the herbicide. The sulfonylurea herbicides exert toxic activity by interfering with the enzyme acetolactat synthase (ALS), which is specific for plants and microorganisms. The enzyme is involved in the synthesis of the branched amino acids leucin, valin and isoleucin. It was shown that the inhibitory effect of metsulfuron methyl could be neutralized by the addition of the amino acids valin, leucin and isoleucin.

NahG and NahW: two different inducible salicylate hydroxylases from *Pseudomonas stutzeri* AN10

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Pseudomonas stutzeri AN10 is a naphthalene degrading strain isolated from highly polluted marine sediments that is able to grow with naphthalene and 2-methylnaphthalene as the only carbon and energy source. The chromosomally coded naphthalene dissimilatory genes of *Ps* AN10 have been sequenced. The gene organization is similar to that of the naphthalene dissimilatory genes of the archetypic naphthalene catabolic plasmid NAH7 from *P. putida* G7. The first operon, consisting of *nahAaAbAcAdBFCE*, codes for enzymes responsible for the transformation of naphthalene into salicylate; and the second one, *nahGTHINLOMKJ*, codes for the conversion of salicylate to pyruvate and acetyl-CoA. Salicylate 1-hydroxylase (*nahG* gene product) catalyzes the transformation of salicylate to catechol, which is further metabolized via a *meta*-cleavage pathway. The regulation of both operons seems to be analogous to that in *Pp* G7 (NAH7): a single protein, NahR, encoded by the *nahR* gene, acts as a positive regulator at both promoters. Anion exchange FPLC of cell extracts of salicylate-grown *Ps* AN10 cells revealed the presence of two different salicylate 1-hydroxylase activities. Both enzymes had a broad substrate range. However, one enzyme exhibited higher activity with methylsalicylates compared with chlorosalicylates, whereas the other enzyme had a relatively higher activity with chlorosalicylates. None of those salicylate hydroxylase activities was observed in extracts of succinate-grown *Ps* AN10 cells. Obviously, both enzymes were inducible.

We have cloned, sequenced and characterized an *EcoRI-EcoRI* 7.1 kb DNA fragment situated downstream of *nahR*. It is of a transposon-like structure: two *pinA*-like open reading frames (*orf*) are flanking another *orf*, *nahW*, whose gene product, NahW (predicted MW 43,570 kDa), shares a 25% of identity in aminoacids with the *Pp* G7 salicylate hydroxylase (NahG) and a 23% with the homologous protein NahG (predicted MW 47,851 kDa) from *Ps* AN10 (*nahG* gene product). Similar genes (*nahG* plus *nahW*) are present in all the *P. stutzeri* strains of our collection, which are able to degrade naphthalene. We have amplified by PCR the coding sequences of *nahG* and *nahW* from *Ps* AN10, and cloned them into pBluescript SK (-) vector under the regulated expression of the *Plac* promoter. Both the NahW and the NahG protein were overproduced, purified and biochemical characterized. NahW is a dimeric protein with an MW of 90 kDa (MW of each subunit is 45 kDa) whereas NahG is a monomer with a MW of 50 kDa. NahG could use both NADH and NADPH as electron donors whereas only NADH was an effective electron donor for NahW. Analysis of the substrate spectrum revealed significant differences: NahG preferences were methylsalicylates over chlorosalicylates, whereas in the case of NahW, chlorosalicylates were the preferred substrates. Those different kinetic properties were already observed for the two salicylate hydroxylases induced during growth with salicylates.

In conclusion, according with our genetic and biochemical data *P. stutzeri* AN10 harbours two different inducible salicylate hydroxylases, NahG and NahW, with different kinetic properties of probably different evolutionary origin.

A functional periplasmic disulfide oxidoreductase in necessary for the expression and proper folding of OprF from *Pseudomonas aeruginosa*

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Correct disulfide bond formation is a prerequisite for native protein folding and stability of disulfide bond containing periplasmic, outer membrane, and secreted proteins. One enzyme involved in this process is the disulfide oxidoreductase DsbA. In *Escherichia coli*, DsbA is a soluble periplasmically located protein which acts as a foldase. DsbA catalytically introduces new disulfide bonds into proteins which are destined for either the periplasm, the outer membrane, or the external environment as they enter the periplasm through the general secretory pathway. Disulfide bond formation in these proteins is not absolutely abolished in strains lacking DsbA, but it is dramatically reduced, demonstrating that DsbA is necessary to encourage proteins to fold properly at a pace which equals their rate of translocation out of the cytoplasm. In addition to donating disulfide bonds to proteins which require them, DsbA has also been shown to have a chaperone function (1).

We are interested in using *P. aeruginosa* as a model system to study the process of disulfide bond formation and substrate specificity of disulfide oxidoreductases in general. *P. aeruginosa* produces several, well characterized proteins which have been shown to contain disulfide bonds. We have used one of these proteins, OprF, which is proposed to contain two disulfide bonds. We show here that, when expressed in a wild type *E. coli* background, OprF is oxidized. However, in a *dsbA* or *dsbB* null background strain of *E. coli*, OprF becomes reduced, demonstrating that disulfide bond formation in OprF is dependent upon the activity of a disulfide oxidoreductase and that *E. coli* DsbA will suffice. This information is interesting in light of the fact that, although DsbA from *P. aeruginosa* has an active site which is homologous to *E. coli* DsbA, these two proteins are only 41% identical. In fact, *P. aeruginosa* DsbA appears to be much more closely related to a disulfide oxidoreductase from *Azotobacter vinelandii* (2) than to *E. coli* DsbA.

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Design of Bacterial Inoculants for the Biodegradation of Polychlorinated Biphenyls (PCBs) in the Rhizosphere

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Bacteria such as *Pseudomonas fluorescens* F113 are ecologically adapted to colonise and compete in the soil rhizosphere environment. Recruitment of new metabolic pathways to modify such bacteria to degrade xenobiotics (in soil) may prove to be a useful strategy to design novel microbial inoculants for the biodegradation of pollutants.

Genes encoding the pathway for the degradation of biphenyl (BP) and the co-metabolism of polychlorinated biphenyl (PCBs) were introduced into *P. fluorescens* F113 to construct the genetically modified strain F113pcb. Expression of the *bph* recombinant genes were detected in the rhizosphere which suggests considerable potential for manipulating the rhizosphere for the bioremediation of pollutants in soil (1).

To expand the degradative potential of F113pcb, the toluene degradative pathway was introduced by transfer of the TOL plasmid, pWWO. Another approach taken to broaden the range of substrates degraded by F113pcb, was mutagenesis with mutagens such as nitrous acid. There is evidence that both these strategies can be successful in generating derivatives of F113pcb with altered degradation properties.

To realise the potential of rhizosphere based bioremediation, it is important to note other compounds in polluted soil which may limit the survival of the inoculant. Heavy metal contamination, in particular, mercury, is common in polluted ecosystems. Therefore, genes encoding the *mer* (mercury resistance) operon were introduced into F113pcb with a view to engineering inoculant strains resistant to this heavy metal.

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Regulation of pyochelin-mediated iron assimilation in *Pseudomonas aeruginosa* 7NSK2

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Pseudomonas aeruginosa 7NSK2 produces three siderophores in response to iron deprivation: pyoverdine, pyochelin and its precursor salicylic acid. As both pyochelin and salicylic acid are involved in the ability of *P. aeruginosa* to control plant diseases, a better understanding of the regulation of these siderophores might result in a more reliable and more consistent biological control.

A *pchD*'-'*lacZ* (*pchD*) is involved in the biosynthesis of pyochelin) fusion was introduced into *P. aeruginosa* 7NSK2 and different siderophore-deficient derivatives to assess pyochelin production. The transcriptional activity of *pchD*'-'*lacZ* was high for pyochelin-producing strains and low in pyochelin-deficient strains. Strains that do not produce pyochelin produced little if any of the 75 kDa ferripyochelin receptor. The presence of pyoverdine diminished the expression of *pchD*'-'*lacZ*, while Zn(II) totally inhibited *pchD*'-'*lacZ* expression and production of the ferripyochelin receptor. Surprisingly, however, pyochelin-mediated iron assimilation genes were induced in mutant MPFM1-569. This pyoverdine-negative mutant is also unable to produce pyochelin and salicylic acid due to a mutation in *pchA*, a gene involved in the biosynthesis of salicylic acid. To further study this phenomenon, the pyochelin-negative, but salicylic acid-positive mutant KMPCCH (mutated in *pchX*, an uncharacterized gene involved in the conversion of salicylic acid into pyochelin) was mutated in *pchA* (KMPCCH-567) to inactivate salicylic acid production. The phenotype of mutant KMPCCH-567 is identical to MPFM1-569. However, *pchD*'-'*lacZ* and the ferripyochelin receptor appeared not to be induced in mutant KMPCCH-567. This indicates that extreme iron limitation in mutant MPFM1-569 is not the major factor contributing to the high expression of *pchD*'-'*lacZ* and the ferripyochelin receptor. KMPCCH-567 was complemented for the *pchX* mutation by introducing clone K5. The resulting strain KMPCCH-567(K5) is phenotypically and genotypically identical to MPFM1-569. Expression of *pchD*'-'*lacZ* and the ferripyochelin receptor was induced in this strain. This suggests that the difference in induction between KMPCCH-567 and MPFM1-569 can be attributed to the *pchX* gene product. Moreover, *pchD*'-'*lacZ* and the ferripyochelin receptor could be induced in mutant KMPCCH by adding filter-sterilized supernatant of an iron-limited culture of MPFM1-569 or an ethylacetate extract of the same supernatant to the culture medium. These results suggest that an ethylacetate extractable, extracellular metabolite of MPFM1-569, which is not pyochelin, can induce the pyochelin-mediated iron assimilation system. The protein encoded by *pchX* is probably involved in the biosynthesis of this metabolite. We are currently investigating the identity of this inducing metabolite.

Steroid induction of β -hydroxysteroid dehydrogenase (β -hsd) gene expression in *Comamonas testosteroni* (ex *Pseudomonas testosteroni*): Transcriptional regulation

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Comamonas testosteroni can grow on a variety of steroid compounds as the sole carbon and energy source. This bacterial strain can effect the complete oxidative degradation of the steroid skeleton by a series of enzymes induced by the presence of these compounds in the culture medium. Some of these enzymes have been isolated, purified and characterized biochemically but very little information is available on the regulation of the genes encoding these degradative enzymes. Previously we have isolated and cloned the gene encoding the β -HSD enzyme responsible of the transformation of testosterone into androstenedione. In the present study we have carried out Northern blot analysis which indicated the presence of a 1 kb transcript inducible with several steroids. This transcript could be detected as early as 30 min after that steroid was added to minimum media. Furthermore, the expression of β -hsd transcript was repressed when the bacteria was grown in the presence of testosterone plus another carbon source such as acetate indicating that β -hsd gene is under catabolite repression. In order to investigate the molecular basis of the β -hsd transcriptional regulation, we performed gel-shift assays. It was found that protein extracts from testosterone-induced *Comamonas testosteroni* cells were able to interact specifically with DNA fragments carrying the β -hsd promoter. Additionally, southwestern blot analysis demonstrated the presence of two different 54 kDa testosterone inducible proteins. Taken together, our results suggest that these DNA binding proteins and their target sequences may be involved in the regulation of β -hsd gene mediated by testosterone.

A Novel Extracellular Aminopeptidase of *Pseudomonas aeruginosa*.

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Previous studies of *P. aeruginosa* proteases have emphasized three secreted endopeptidases, elastase (encoded by *lasB*), alkaline proteinase, and LasA. Based on leucine p-nitroanilide (pNA) hydrolysis, we demonstrated an aminopeptidase activity in culture filtrates of *P. aeruginosa* strains FRD2 (wild type), FRD2128(Δ lasA), and FRD740(Δ lasB). The aminopeptidase was partially purified from culture filtrates of both mutant strains by DEAE-cellulose chromatography and found to be heat resistant (70°C; 1-3h). The molecular mass of the enzyme was ~56 kDa. Heat treatment of aminopeptidase preparations from strain FRD2128 but not FRD740, led to the conversion of the 56 kDa enzyme to a ~28 kDa protein which retained enzyme activity. Heating was also associated with the disappearance of most other proteins, and thus, served for further enzyme purification. Conversion of the 56 kDa enzyme to its smaller form was inhibited by phosphoramidon. In addition, when heated in the presence of elastase, the 56 kDa enzyme from strain FRD740(Δ lasB) was degraded into the 28 kDa form, suggesting a role for elastase in this process. The pH optimum for Leu-pNA hydrolysis was ~8.5 and the activity was inhibited by dithiothreitol, excess Zn^{+2} , and zinc chelators, but not by serine-proteases inhibitors, N-ethylmaleimide, and phosphoramidon. Leu-pNA was cleaved at a rate one to two orders of magnitude higher than those of the respective Met, Ala, Pro, Val, and Phe derivatives, while no hydrolysis of Gly- or Glu-pNA was evident. In general, *Pseudomonas* aminopeptidase properties were analogous to those of *Streptomyces griseus* and *Aeromonas proteolytica* aminopeptidases, suggesting that all three peptidases might be related. The role of this newly discovered aminopeptidase is not yet defined but it may well contribute to *P. aeruginosa* virulence by extending endopeptidases action on host proteins.

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Mango trees (*Mangifera indica* L.) were recently introduced in Southern Spain. This is a high-added value crop with good market perspectives, and the cultivation area is rapidly increasing. Bacterial apical necrosis, caused by *Pseudomonas syringae* pv. *syringae* (1), is a major disease of mango trees in the Spanish fields that is severely limiting the production of fruits. Farmers usually rely on the use of copper compounds to control the disease, albeit with a limited success. With the aim to develop better control strategies, we have undertaken the genetic characterization of *P. syringae* pv. *syringae* strains isolated from healthy and diseased trees in different locations of the mango growing areas. Around 62% of the isolates were resistant to copper compounds, with in vitro CMI values ranging from 0.8 to 1.6 mM. The plasmid profile of the isolates was variable, and did not show any obvious correlation with the copper resistance (Cur) phenotype of the isolates. Most (ca. 60%) of the strains analyzed contained a plasmid band of 65 kb; and a third of these showed an additional band of 88 kb. The rest of them either contained no plasmids, or showed a faintly visible band of ca. 150 kb. We performed hybridization analyses using the copper resistance genes *copJ*, from *P. syringae* pv. *syringae* (2), and *copABCD*, from *P. syringae* pv. *tomato* PT23 (3). All the strains examined showed medium to strong homology to *copJ*, irrespective that they were Cur or copper sensitive (Cus), which suggest that in these strains *copJ* could be involved in functions other than copper resistance. On the other hand, *copABCD* showed hybridization with the 65 kb and the 150 kb plasmids from Cur strains, while there was no hybridization with total DNA from Cus strains. Some of the Cur strains also showed an additional hybridization band located in the chromosome. Restriction and hybridization analysis of the 65 kb plasmids from several Cur and Cus strains showed that they are identical among the Cur strains and highly related to the ones from Cus strains. By Southern hybridization analysis using the origin of replication from plasmid pPT23A as a specific DNA probe, we determined that the 65 kb plasmids belong to the family pPT23A-like (4, 5). The characteristics of 65 kb plasmid were similar from isolates with only this plasmid and from isolates with two plasmids after curation of other plasmids present. Plasmids of this family are widespread among *P. syringae* pathovars and were shown to contain diverse genes that could be involved in pathogenicity or epiphytic survival.

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The production of several virulence factors by *P. aeruginosa* is controlled according to cell density through two complete quorum sensing systems, LasRI and VsmRI. We have shown the existence of a regulatory cascade in which the Las system controls at a transcriptional level the *vsm* genes. Moreover, Vsm controls the expression of a critical gene such as *rpoS* encoding a sigma factor specific for regulation of genes expressed in the late phase of growth.

Most of the virulence factors, including elastase and exotoxine A, are secreted throughout the Xcp general secretion pathway. The two evidenced quorum sensing systems are involved in *xcp* genes regulation and their effect is cumulative. Thus, the two regulatory systems coordinate the synthesis of virulence factors and the expression of the secretion machinery.

The control by Vsm of a global regulator such as RpoS and of a global process like the general secretion pathway adds greater importance to the *vsmR* regulation. Beside the Las dependent regulation, other controls recently appear to modulate *vsmR* expression. We have shown that regulatory *alg* genes affect *vsmR* expression. Moreover, DNA gel shift assay experiments show that another regulator acts on *vsmR* promoter. Taken together, these results highlight the complexity of *vsmR* regulation in particular and of quorum sensing system in general.

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In situ studies of TOL-plasmid (pWWO) spread were performed in a seven species model community using the green fluorescent protein (GFP) as a plasmid marker. It was found that TOL-plasmid could only establish in the dominant community member. *P. putida* RI. To specifically follow the appearance of transconjugants the TOL plasmid was tagged with the *gfp*-gene fused to the hybrid *lac* promoter PA1/O4/O3 and a specific donor strain was constructed with the *lacI*-gene inserted on the chromosome. This design prevent expression of GFP in the donor cells, whereas transconjugant cells would be monitored as green fluorescent cells upon illumination with blue light.

Biofilms were established in flow chambers with benzylalcohol as the sole carbon source, and two days after the initial colonization of the seven species, donor cells were introduced. Time course analysis of the population distribution of transconjugants, donor and recipient cells collected from the flow chamber effluents showed that the fraction of donor cells in the following 5 days after introduction decreased 1 order of magnitude, whereas the fraction of transconjugants increased exponentially in the same period of time. We took this as an indication that real donor-to-recipient transfer events only in short period after introduction had a significant influence on transconjugant accumulation, after that further spread of the transconjugants was caused mainly by vegetative growth due to an obtained growth advantage over the non-infected recipient cells.

On-line studies of the community structure showed a high degree of heterogeneity in the spatial distribution of transconjugants in the biofilm. Donor cells introduced at 10⁶ cfu/ml resulted in a number of hot spots in the biofilm with high density of transconjugants. A hot spot region would appear as a strong green fluorescent (transconjugant infected) colony with other green fluorescent colonies infected downstream in the media flow direction but not upstream the colony. We speculate that a real donor-to-recipient transfer event initially had occurred in this colony and that the primary transconjugants eventually proliferated to create the fluorescent colonies.

By employing scanning confocal laser microscopy on fixed biofilms where all *P. putida* cells were identified by *in situ* hybridization using a 16S RNA *P. putida* specific probe, the green fluorescent transconjugants were found always to be associated with non-infected *P. putida* RI recipient micro-colonies as cell layers covering the micro-colony. Pure colonies of transconjugants were not observed, indicating that transconjugant proliferation is highly dependent on the existing pool of *P. putida* RI cells. This was further evidenced in time course experiments where the donor, recipient and transconjugant distribution were followed in flow experiments with reduced number of *P. putida* RI cells in the biofilm.

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Production of the extracellular metabolites pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, and hydrogen cyanide (HCN); an extracellular protease; and tryptophan-side-chain oxidase (TSO) are controlled by the *apdA* (= *lemA*) and *gacA* genes, which encode a sensor kinase and response regulator, respectively, in *Pseudomonas fluorescens* Pf-5. Because the phenotypes of *ApdA*- and *GacA*- mutants commonly are identical, *ApdA* and *GacA* are likely to be partners comprising a classical two-component regulatory system, which together regulate transcription of target genes (1,3,4,5). For example, transcription of pyoluteorin biosynthesis genes was low in *ApdA*- and *GacA*- mutants in comparison to the wildtype strain Pf-5. Colonies with a *ApdA*-/*GacA*- morphology (large, orange, and highly fluorescent) arise with remarkable frequency in cultures of *P. fluorescens* (2,3); they comprised up to 40% of the total colonies harvested from six-day cultures of Pf-5 grown in nutrient yeast broth. Certain colonies with the altered morphology exhibited only a subset of the phenotypes controlled by these global regulators. For example, a Pf-5 derivative with a single point mutation in the carboxy-terminal region of *GacA* did not produce pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol or protease, but did produce HCN and TSO at less than wildtype levels. The point mutation was located outside of known functional domains and resulted in a conservative substitution from alanine to valine in *GacA*. Thus, the carboxy-terminal region of *GacA* may confer specificity for certain target genes.

In previous studies, pleiotropic mutants with altered colony morphology that arise upon prolonged culture of *P. fluorescens* have been designated as *ApdA*⁻ or *GacA*⁻ mutants following complementation with the respective wildtype genes. In Pf-5, wildtype *apdA* and *gacA* genes cloned in low copy number plasmids such as pRK415 (1-4 copies/cell) restore the wildtype phenotype to *GacA*⁻ and *ApdA*⁻ mutants, respectively. Our preliminary results indicate, however, that when present on a high copy plasmid (i.e. pME6000, 16-20 copies/cell), *apdA* does not complement either *ApdA*⁻ or *GacA*⁻ mutants and *gacA* complements both *ApdA*⁻ and *GacA*⁻ mutants. We speculate that proper function of the *ApdA*/*GacA* system requires a stoichiometric balance between the sensor kinase and the response regulator that is disrupted by high copy numbers of either *apdA* or *gacA*.

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The biodegradation of different chlorobenzoates has been studied in three *Pseudomonas* strains, *P. aeruginosa* 142 (1), *P. aeruginosa* JB2 (2) and *P. putida* P111 (3). Chlorobenzoate utilization was measured in minimal media (MM) and in MM supplemented with glucose (MMG). In sugar supplemented media the duplication time was considerably decreased, the initial lag time in chloride release shortened, and the chloride release was increased. As a result, higher rates of chlorobenzoate transformation were achieved in less time compared to cultures using this compounds as the sole carbon source. This suggests the lack of catabolic repression, at least for the upper metabolizing pathway. In glucose supplemented media the *P.* strains were able to partially metabolize several chlorobenzoates which could not themselves support growth such as 2,3- and 2,6-chlorobenzoate. Accumulations of intermediates was observed under these conditions. Chlorocatechol, and 3- and 4- chlorocatechol dioxigenase activity were determined for each case, showing independent induction. Differences in substrate utilization are explained by the specificity of initial aromatic ring dioxigenase, the ring-cleavage dioxigenase and the induction of the key enzymes in the catabolic pathway. The results obtained in fast-growing cultures are compared with those published for cells using these compounds as energy and carbon source.

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A number of traits contributing to root colonisation by fluorescent *Pseudomonas* have been identified (de Weger *et al.*, 1995) but the picture is far from complete. For some *P. fluorescens* strains, it was shown that colonisation ability is impaired in non-motile mutants (de Weger *et al.*, 1987; Simons *et al.*, 1996). However the role of chemotaxis in efficient root colonisation has not been established. Chemotaxis allows bacteria to sense, move towards and eventually assimilate plant-exuded nutrients. Methyl-accepting chemotaxis proteins (MCPs) are membrane bound receptors linked to a complex intracellular signal transduction machinery which steers flagellar movement of cells following MCP-mediated perception of stimuli (Garrity and Ordal, 1995).

P. fluorescens OE28.3 was isolated from wheat rhizosphere and has been shown to efficiently colonise roots of various plants (De Mot and Vanderleyden, 1991). Using the MCP gene *pcIA* recently isolated from a *P. aeruginosa* strain (Kuroda *et al.*, 1995), a number of MCP-like genes have been isolated from the *P. fluorescens* OE28.3 genome and sequenced. These genes show homology to a number of known MCP proteins, exhibiting the well conserved motifs of MCP proteins.

In order to evaluate what role MCPs have in the chemotactic response of *P. fluorescens* OE28.3, insertional activation of the isolated gene will be performed. This will allow assessment of the role each individual gene has on performance in the rhizosphere.

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Influence of soil type and *Zea mays* cultivar on association between *Burkholderia cepacia* and maize roots

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The understanding of the influence of environmental factors on the size and composition of rhizosphere bacterial populations is a pre-requisite for a wider implementation of rhizobacteria for plant growth promotion and biological control.

Bacteria belonging to the species *Burkholderia cepacia* seem to be closely associated with *Zea mays* roots, where they can repress soilborne fungal pathogens species and exert positive effects on plant growth. The aim of this work was to study the influence of soil type and maize cultivar on *B. cepacia* populations associated to roots of maize plants cultivated in different regions of Italy. Three fields located in northern, central and southern Italy, respectively, were chosen and two different cultivars of maize were cultivated in each of them. Plants of each cultivar were collected from each field after 20-40 days of plant growth. Roots were excised from plants and loosely adhering soil was removed. A portion of each root was blended and resuspended in phosphate buffered saline (PBS) and serial dilutions of this suspension were plated on the semi-selective PCAT medium (1) in order to estimate microorganisms belonging to the *B. cepacia* species and to isolate some of them for further analysis. The assignment of isolates to the species *B. cepacia* was performed by means of amplification and restriction analysis of 16S rDNA (ARDRA) by using the restriction enzyme *AluI* (2). By this method we observed that microorganisms belonging to the *B. cepacia* species were present in large amount in the rhizosphere of maize plants collected from all the fields examined. Analysis of genetic diversity in each *B. cepacia* population has been performed by means of Random Amplified Polymorphic DNA technique (3) to assess the influence of soil type and/or plant variety on microbial biodiversity.

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Identification and characterization of a *Sphingomonas* sp. strain AD109 gene cluster required for the specific desulfurization of dibenzothiophene

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Biocatalytic desulfurization (BDS) is a process that is being developed based on naturally occurring bacteria that can remove organically bound sulfur from petroleum without degrading the fuel value of the product. In order to gain a better understanding of the distribution of genes encoding the sulfur specific oxidation of dibenzothiophene (DBT) in the environment, enrichments with oil contaminated soil were performed in a minimal glucose medium using an intermediate in DBT desulfurization, 2-(2-hydroxyphenyl)-benzenesulfinate (HPBS), as the sole sulfur source. After repeated liquid culture enrichments, a pure isolate, designated AD109, was identified that can also use DBT and DBTO₂ as sole sulfur sources for growth. Based on fatty acid analysis, this microorganism was tentatively identified as a *Sphingomonas* species. The genes responsible for DBT desulfurization (*dsz*) by strain AD109 (*i.e.*, the conversion of DBT to 2-HBP and sulfite) have been cloned. DNA sequence analysis has revealed that the organization of the *Sphingomonas* AD109 *dsz* genes is identical to that found in *Rhodococcus erythropolis* strain IGTS8 (*i.e.*, *dszA*, *B*, *C*). The proteins encoded by these genes also have significant homology to the IGTS8 DBT monooxygenase (DszC), DBTO₂-monooxygenase (DszA) and HPBS desulfinase (DszB) (76, 66, and 67% identity, respectively). Furthermore, Western blot analysis has shown that the AD109 Dsz proteins are antigenically similar to the *Rhodococcus* counterparts. The Dsz protein catalyzing the last step in the pathway, HPBS desulfinase, has been partially purified (Mr = 40,000) and the N-terminal amino acid sequence of this protein is identical to the N-terminal residues predicted from the AD109 *dszB* nucleotide sequence. In addition, based on the kinetic parameters calculated from the enzyme progress assay the catalytic rate of the *Sphingomonas* HPBS desulfinase is comparable to that from *Rhodococcus* IGTS8. These results provide the first genetic evidence that naturally occurring Gram-negative species are capable of specific desulfurization of DBT.

Bacteriophage $\phi 6$ is a lipid envelope containing dsRNA virus of *P. syringae*. It is known, that upon the infection phage $\phi 6$ attaches to the host pilus, virion nucleocapside (NC) penetrates into the bacterial intermembrane space by fusion of the phage envelope with the cellular outer membrane (OM) and transfers through the host plasma membrane (PM) via membrane invagination and an intracellular vesicle [1]. We studied changes in amount of lipophilic ions tetraphenylphosphonium (TPP⁺) and phenyldicarbonyldecaborane (PCB) bound by *P. syringae* as well as the intracellular ATP and K⁺ content of the cells during the phage penetration. Ion concentrations in the medium were monitored by the selective electrodes. Intact cells bind only small amounts of TPP⁺ and PCB because the OM of Gram-negative bacteria such as *P. syringae* is low permeable to lipophilic compounds. Addition of EDTA caused an influx of TPP⁺ into the cells due to an enhancement of the OM permeability and allowing the distribution of this cation between the cytosol and external medium according to the membrane voltage. In contrast to *Salmonella* or *Escherichia* cells the OM of *P. syringae* was sensitive to EDTA not only in Tris but also in phosphate buffer. Such treatment considerably increased the amount of PCB bound by the cells and induced a leakage of intracellular K⁺. The experiments with the bacterial spheroplasts showed that PM of *P. syringae* is rather permeable to ATP. However, the permeability was reduced considerably if apyrase was hydrolyzing ATP outside the cells.

Three steps of phage $\phi 6$ -induced changes in the cell envelope permeability could be defined at the early stages of infection. Permeabilization of the OM to lipophilic compounds was followed by depolarization and subsequent repolarization of the PM. The duration of these steps correlated well with the time intervals required for the interaction of phage particles with the successive layers of the host envelope [1]. However, the stage of depolarization was induced more rapidly if the OM of *P. syringae* was permeabilized by EDTA before the infection. The extent of depolarization was depended on the metabolic activity of the cells and their capability to repolarize the PM after the phage treatment. Phage particles possessing a heat-inactivated peptidoglycan-degrading enzyme P5 enhanced the permeability of the cellular OM to lipophilic compounds, but the PM depolarization and repolarization were not observed. The spheroplast infection by NCs showed that membrane voltage is necessary for the formation of intracellular vesicle. Low intensities of $\phi 6$ -induced binding of PCB, leakage of the intracellular K⁺ and depolarization of the PM indicate that the structural and functional integrity of the cellular PM is not affected dramatically during the NC penetration.

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Pseudomonas aeruginosa utilizes a mechanism known as quorum sensing to regulate a number of virulence genes. The first quorum sensing system discovered in *P. aeruginosa* involves an autoinducer, termed PAI-1, and a transcriptional activator protein, LasR. The gene encoding LasR and the gene for the autoinducer synthase, *lasI*, are separated by a 365-bp intergenic region. Novel promoter activity antisense to *lasR* was recently discovered originating from within the *lasR/lasI* intergenic region. Expression of this transcript, called *rsaL*, repressed expression of a number of the virulence genes controlled by LasR/PAI. Several plasmids containing *rsaL* and *lasR* under native or *lac* promoters were examined in PAO-R1 (*lasR*). LasR/PAI-1-dependent gene activation was monitored using a *lasB-lacZ* fusion on each plasmid. Increased expression of *rsaL*, relative to *lasR*, resulted in repression of *lasB* expression. Analysis of plasmids containing *lasR* alone, or *lasR* and *rsaL* in *E. coli lasB-lacZ* and *lasI-lacZ* λ lysogens revealed that RsaL directly affects *lasI* expression. Cloning *rsaL* into an expression vector that attaches six histidine residues to the amino terminus of the protein allowed purification of RsaL on Ni-NTA resin.

A diversity study of pseudomonads based on two lipoprotein genes, *oprI* and *oprL*

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The gene corresponding to *oprI* (1) was PCR-amplified from different fluorescent pseudomonads and the products directly sequenced on an ALF automatic sequencer (ALF, Pharmacia). The sequence was extremely conserved among *P. aeruginosa* isolates but showed some divergences for the other fluorescent pseudomonads. Synonymous as well as non synonymous mutations were detected in the *oprI* sequence. Comparison of the different *oprI* sequences allowed us to assess the diversity between and within fluorescent pseudomonads species. It confirmed that *P. aeruginosa* form a rather homogenous group while *P. fluorescens* demonstrated a high degree of heterogeneity. The ecological origin of the *P. fluorescens* strains did not correlate with the obtained clusters. The complete *oprL* gene coding for the peptidoglycan-associated lipoprotein (PAL) of *P. aeruginosa* (2) could only be amplified from *P. aeruginosa* isolates while the 3' half of the gene could be amplified from different fluorescent pseudomonads.

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A site-specific recombinase is required for root colonization of *Pseudomonas fluorescens* strain WCS365

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A novel colonization mutant of the efficient root colonizing biocontrol strain *Pseudomonas fluorescens* WCS365 is described. After co-inoculation of sterile potato plantlets with a 1:1 mixture of cells of wildtype and mutant strain PCL1233, followed by incubation for 14 days in a gnotobiotic quartz sand/plant nutrient solution system, the mutant is at least 50-fold (but usually more severely) impaired in its ability to colonize the potato root tip. Mutant PCL1233 is also impaired in its ability to colonize the roots of radish, wheat and tomato, indicating a broad host range mutation. The colonization of the mutant is also impaired when studied in potting soil, indicating the importance of the defective gene in agriculture. A DNA fragment which is able to complement the mutation for colonization revealed a multicistronic transcriptional unit comprising at least six ORFs (open reading frames) of which the last one is largely incomplete and therefore is unlikely to play a role in complementation. The ORFs have the following order of transcription and exhibit similarity to *lplL* (encoding a lipopeptide), *lysA*, *dapF* (both involved in lysine biosynthesis), *orf235/233* (unknown function), *xerC/sss* (a site-specific recombinase), and *orf238* (unknown function), respectively. The transposon insertion in PCL1233 appeared to be present in the *orf235/233* homologue, designated *orf240*. A mutation introduced in the *xerC/sss* homologue, designated *sss*, revealed that the *sss* gene homologue rather than *orf240* is crucial for the ability of mutant PCL1233 to colonize the root tip. *xerC* in *E. coli* and *sss* in *P. aeruginosa* encode proteins which are members of the lambda integrase family of site-specific recombinases. These include *xerC*, *xerD*, *sss*, *fimB* and *fimE*, and are implicated to play a role in various important processes of bacterial cells such as phase variation of a DNA element involved in the synthesis of fimbriae. The function of the *sss* site specific recombinase in colonization is discussed in terms of genetic rearrangements which are involved in the generation of different phenotypes, a process which is assumed to allow a bacterial population to occupy various ecological niches. Mutant PCL1233 is assumed to be locked in a phenotype that is not well suited to compete for colonization in the rhizosphere. This is the first report which shows the importance of phase variation for rhizosphere competence.

In biocontrol experiments using a tomato-*Fusarium oxysporum* f. sp. *radicis lycopersici* system inoculation of seeds with cells of *P. fluorescens* WCS365 significantly increases disease suppression. The observation that the *sss* mutant PCL1233 has decreased biocontrol properties proves for the first time the importance of colonization for biocontrol. Introduction of the involved colonization operon in the poor colonizer *P. fluorescens* WCS307 and in the good colonizer *P. fluorescens* F113, increased the competitive colonization ability on the tomato root tip of these strains 16 to 40-fold and 8 to 16-fold, respectively. This introduction also increased the biocontrol activity of *P. fluorescens* WCS307. The latter results show that improvement of colonization and biocontrol of wild type *Pseudomonas* strains by genetic engineering is a realistic goal.

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Pseudomonas aeruginosa is an adaptable bacterial saprophyte that can cause opportunistic infection in humans, animals, insects and plants. Infection by *P. aeruginosa* is often associated with mortality in patients with cancer, burns wounds and organ transplants and is the leading cause of lung infection and death in sufferers of cystic fibrosis. Isolation of *P. aeruginosa* has been reported from a variety of sources including clinical environments, personnel and patients (1), water (2), soil (3), plants (4) and from a gasoline contaminated aquifer (5). Although the organism is thought to be ubiquitous within the natural environment, recovery of *P. aeruginosa* isolates is often at low frequency.

Here we report on the finding of low numbers of *P. aeruginosa* within soil and water environments compared with a high frequency of isolates recovered from a mushroom growing unit. By comparing the flagellin gene sequences from two *P. putida* strains and a previously published *P. aeruginosa* strain, primers specific to the N-terminal (CW46: GGCTGCAGATCNCCAA) and C-terminal (CW45: GGCAGCTGGTTNGCCTG) conserved regions of *Pseudomonas* spp. flagellin genes were designed (6). Using these primers the central variable region of the flagellin gene from a number of different rRNA group 1 pseudomonads can be amplified. Environmental isolates of *P. aeruginosa* were compared to clinical isolates by PCR-RFLP analysis of the flagellin gene. This method has differentiated 20 RFLP groups of *P. aeruginosa* and has been used, in conjunction with Pulsed Field Gel Electrophoresis, to provide the first molecular evidence of the transmission of a β -lactam resistant strain of *P. aeruginosa* amongst CF patients attending a clinic in the UK (7). PCR-RFLP analysis of the flagellin gene of 60 environmental isolates of *P. aeruginosa* showed that although some novel genotypes were identified amongst the environmental isolates, the majority of the strains corresponded to the largest flagellin gene RFLP group found in a previous study of clinical isolates (6). These results lend support to growing concerns that discrimination between potentially pathogenic strains of *P. aeruginosa* and those which might be considered harmless, may not be possible. Furthermore, the mushroom growing unit with conditions of elevated temperature, humidity and nutrients was found to offer an environment in which *P. aeruginosa* can thrive and as such it should be added to the growing list of environmental 'hosts' in which this organism can be found.

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Transposon mutagenesis is an effective method of generating insertion mutations in target DNA. Transposon mutagenesis is especially useful in bacterial species with poorly described genetic systems or inadequate molecular tools. A recent development in the use of transposons as molecular tools has been the placement of the transposase outside of the transposon's terminal inverted repeats. After transposition from a suicide plasmid and insertion into the target DNA, mini-transposons (mini-Tns) do not retain their cognate *cis*-oriented transposase. This produces a stable insertion and allows for multiple rounds of mutagenesis using mini-Tns containing different selectable markers. In order to extend the utility of the basic mini-Tn, novel genetic elements have been constructed which consist of a conditional origin of replication within a mini-Tn. Elimination of the plasmid origin of replication from the suicide delivery vector requires the mini-Tn element to serve as the suicide delivery plasmid. Therefore, this plasmid:mini-transposon, or "plasposon", can not exist in the absence of either the plasmid DNA or the mini-Tn. Upon transposition of the plasposon's mini-Tn into a target DNA, the plasposon's transposase and origin of transfer form a non-replicating DNA circle and are lost from the cell population. Subsequent *in vitro* digestion of the chromosomal DNA containing the integrated mini-Tn with a restriction endonuclease, followed by self-ligation and the transformation of an appropriate *E. coli* host, produces a plasmid which contains chromosomal DNA flanking the mini-Tn's site of insertion. These plasposons were engineered to contain rare restriction endonuclease sites which permit the recovery of large DNA clones. In combination with Pulse Field Gel Electrophoresis, these rare restriction sites can also be used to localize the mini-Tn's point of insertion to a specific DNA fragment on a physical genome map. The modular structure of plasposons allows for the simple exchange of antibiotic resistance cassettes, permitting selection based on the antibiotic resistance characteristics of the target bacteria.

DEVELOPMENT OF ENGINEERED *E. COLI* BASED ON *PSEUDOMONAS* OXYGENASES FOR BIOCONVERSION OF SUBSTITUTED AROMATIC COMPOUNDS.

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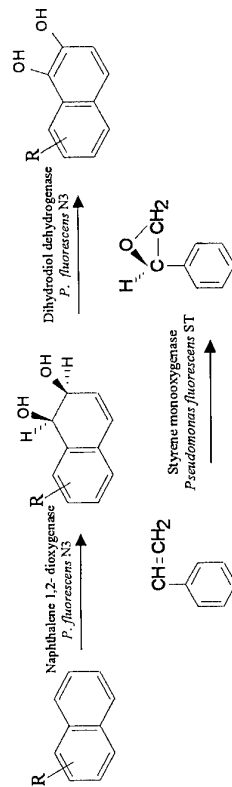
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The development of biotransformation processes for large scale production of useful compounds with high regio- and stereoselectivity is increasing interest in microbial technology. Our group is interested in the study and applications of oxygenases with relaxed substrate specificity produced by *Pseudomonas* strains, able to oxidize different aromatic hydrocarbons in hydroxylated compounds and epoxides. To obtain recombinant strains producing these metabolites we cloned the naphthalene dioxygenase and the dihydrodiol dehydrogenase genes from *Pseudomonas fluorescens* N3 and the styrene monooxygenase from *Pseudomonas* ST.

The naphthalene dioxygenase and the dihydrodiol dehydrogenase genes were localized on a 4.3 Kb *HindIII*-*ClaI* and a 2.8 Kb *BamHI* fragments respectively and cloned in *E. coli* JM109. By cloning of the *HindIII*-*ClaI* fragment carrying dioxygenase gene including all the sequences for its expression and the regulatory region localized on the 3.5 Kb *HindIII* fragment, we obtained a recombinant strain JM109(pPS1778) inducible by salicylic acid. This recombinant strain was analyzed as a biocatalytic tool to produce dihydrodiols from substituted aromatic compounds. The dihydrodiols are produced with high yields, that in some cases correspond to a complete bioconversion of substrate, depending on the nature and the position of the substituent on the aromatic ring. By using recombinant strains containing both the naphthalene dioxygenase and the dihydrodiol dehydrogenase it is possible to obtain catechols with yields of 50%.

Another interesting system is the recombinant clone carrying styrene monooxygenase localized on a 3 Kb *PstI*-*EcoRI* fragment of *P. fluorescens* ST, able to produce the corresponding epoxides from different compounds with a vinyl group. The epoxides produced have an enantiomeric purity of 98%, showing the interest for this monooxygenase in bioconversion processes.



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Isopropylbenzene Catabolic Pathway in *Pseudomonas putida* Kt204: Sequence Analysis of the *ipb* Operon and Surrounding DNA from pRE4

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Pseudomonas putida RE204 employs a set of plasmid-specified enzymes in the catabolism of isopropylbenzene (cumene) and related alkylbenzenes. Strain RE204 and its mutant and recombinant derivatives have been used previously to study the isopropylbenzene catabolic pathway (4) and the biotransformation of various substrate analogs including 6-methylfulvene (3) and benzothiophene (2), and as sources of regulatory elements for the construction of bioreporter strains responding to hydrophobic pollutants (5). A 21,768 bp segment of the plasmid pRE4, which includes the *ipb* (isopropylbenzene catabolic) operon as well as associated genetic elements, has been sequenced; features of that sequence are described here. The *ipb* operon, *ipbAaAbAcADBCGEFHD*, encodes enzymes catalyzing the conversion of isopropylbenzene to isobutyrate, pyruvate, and acetyl-coenzyme A as well as an outer membrane protein (IpbH) of uncertain function. These gene products are 75 to 91% identical to those encoded by other isopropylbenzene catabolic operons (1) and are somewhat less similar to analogous proteins of related pathways for the catabolism of mono-substituted benzenes such as toluene and biphenyl.

ipbR, 3278 bp upstream of *ipbAa*, encodes a positive regulatory protein which has about 56% identity to XylS regulatory proteins of TOL (xylene/toluene) catabolic plasmids. This similarity and that of the DNA sequence in the proposed *ipb* operator-promoter region (*ipbOP*) to the same region of the *xyl meta* operon (*xylOmPm*) suggest that, although the *ipbR* and *XylS* regulatory proteins recognize very different inducers, their interactions with DNA to activate gene expression are similar. Upstream of *ipbR* is an 1196 bp insertion sequence, IS1543, related to IS52 and IS1406 (65% and 66% DNA sequence homology, respectively). IS15406 was recently shown to be located downstream of the closely related cum operon of *P. fluorescens* IP01 (1) although it is not present at the same location in pRE4. Four additional, tightly clustered DNA elements identified by their homology (60 to 80%) to previously described DNA elements occupy 2969 bp of the 3278 bp separating *ipbR* from *ipbAa*. The first of these elements is a 127 bp segment having homology to short DNA segments bordering the upper naphthalene catabolic operon of the plasmid NAH7. It is followed by IS1544, homologous to IS1543, IS1406, and IS52; IS1545, homologous to IS1240; and IS1546, homologous to IS1491. The latter three elements are large fragments of insertion elements and lack inverted repeats and complete transposase genes. The role of these IS elements in the assembly of pRE4 is not known. The locations of IS1543, IS1544, and the related insertion sequence of *P. fluorescens* IP01, IS1406, suggest that they could have formed composite transposons incorporating either *ipbR*, or the *ipb* operon, or both. The 18,492 bp comprising the *ipb* catabolic genes and the other genetic elements are bordered by two identical, directly repeated 1007 bp DNA segments. Homologous recombination between these segments is probably responsible for the occasional deletion of the intervening DNA from pRE4.

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Monoclonal antibody (MAb) directed against the species- specific *B. pseudomallei* protein, the 42- kilodaltons (kDa) protein, were used to monitor cloning and expression of the gene from a *B. pseudomallei* genomic library. The structure of the gene was analyzed, and recombinant protein was produced in *Escherichia coli*. A DNA sequence analysis of the 42-kDa gene revealed the presence of an open reading frame that encoded a protein having 375 amino acid residues and a calculated molecular weight of 40 kDa. Our results provide a basis for further biochemical analysis and immunostimulatory study of the 42 kDa protein, investigation of the role of this protein in host- pathogen interactions and development of rapid and specific diagnostic system for melioidosis.

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PHENOTYPIC AND GENOTYPIC CONSERVATION OF FLUORESCENT PSEUDOMONADS INVOLVED IN THE BIOCONTROL OF *PYTHIUM* SPP.

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Fluorescent pseudomonads have shown considerable promise for use as agents for the control of soil-borne fungal pathogens. However, the mechanisms by which this occurs are poorly understood. The production of various antibiotics and siderophores have been implicated but do not account for all anti-fungal activity observed. Cluster analysis of whole cell fatty acid methyl ester (FAME) composition of *Pseudomonas* strains from around the world revealed distinct groups of strains which were active against *Pythium*, non-active or plant-deleterious. Furthermore, the presence of high proportions of certain cellular FAMEs could be used as molecular markers to identify pseudomonad isolates with high anti-fungal activity. Non-active mutants showed a reduction in the proportion of these FAMEs.

RFLP analysis of conserved regions of the genome such as the ribosomal RNA operon were undertaken. The strains active against *Pythium* clustered within a close taxonomic group implying that a degree of genetic specialisation is required by pseudomonads for successful anti-fungal activity. Indeed, strains capable of controlling *Pythium* may have evolved from a common ancestor or converged due to the high selective pressure apparent in plant-associated environments. These results suggest that there are common genetic and phenotypic traits shared by highly active strains that are important for biocontrol efficacy. Further comparisons will identify other features, which may include a high metabolic rate in soil, aggressive colonisation of plant surfaces or activation by the presence of the fungal pathogen. These can then be screened for, greatly improving the search for new biological control agents. Identification of such traits will also allow better management of the application of BCAs and therefore improve the predictability of their performance in the field.

Plasmid Enhanced Survival of a Fluorescent *Pseudomonad* - A Study of the Factors Involved

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It is presumed that plasmids have a negative impact on their host's fitness under non-selective conditions. However, we have identified a conjugative plasmid isolated from river epilithon, which enhances the survival of a fluorescent *pseudomonad* - *Pseudomonas putida* UWC1 - under low nutrient conditions. To investigate survival in such an environment, starvation conditions were generated by diluting washed cultures of UWC1 (with and without plasmid pQKH6 (Hg^r, Tra⁺, 71kb)) in water and then monitoring the cell survival over a thirty day period. Microcosms inoculated with stationary phase cells maintained culturability at or above 98%. However, the viability of exponentially growing plasmid free cultures was reduced to less than 60%. In contrast, UWC1 carrying pQKH6 maintained culturability at the level of ~ 98% of the initial inoculum irrespective of whether inocula were prepared from stationary or mid-exponential phase cultures. The role of this plasmid in promoting host cell survival was highlighted by studies with other plasmid isolates, including pQKH54 which shares homology to pQKH6. pQKH54 reduced the survival of UWC1 by several orders of magnitude under all conditions. Investigations to identify the plasmid mediated survival factor are underway using naturally occurring deletion derivatives and transposon mutants of pQKH6.

The enhanced survival observed in the presence of the plasmid suggests that plasmid mediated host survival is an important factor in the environmental persistence of particular bacteria and may potentially promote plasmid transfer and maintenance during conditions of stress such as starvation.

Isolation and characterisation of a pleiotrophic mutant of *Burkholderia cepacia* deficient in siderophore production

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Burkholderia cepacia is a Gram negative non-sporing motile rod widely distributed in the environment. It was originally isolated as a phytopathogen but has now found status as an opportunistic pathogen, particularly in cystic fibrosis (CF) patients. The virulence factors of this organism have not been fully determined, though several candidates such as lipase, protease and siderophores have been proposed. Siderophores are low molecular weight iron chelating compounds produced under iron depleted conditions. They enable bacteria to effectively compete with host iron binding proteins, thus allowing bacteria to establish and maintain infections. Production of pyochelin, a green-yellow siderophore specified by many *B. cepacia* CF isolates, has been directly linked with morbidity and mortality in *B. cepacia* infected CF patients. We describe the isolation of a pyochelin deficient mutant (Pch⁻) of a *B. cepacia* CF isolate following transposon mutagenesis with a mini-Tn5 derivative. Conjugation experiments involving an *E. coli* donor strain harbouring the mini-Tn5 transposon promoted efficient mutagenesis of *B. cepacia*. Transconjugants were screened for the absence of fluorescence on iron-limited media. One Pch⁻ mutant was isolated which upon further characterisation exerted pleiotrophic effects, including increased resistance to certain antibiotics, decreased protease secretion, impaired motility, and a defect in the regulation of exopolysaccharide production.

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Burkholderia cepacia is a Gram negative non-spore forming motile rod widely distributed in the environment. Originally isolated as a phytopathogen it has recently found status as an opportunistic pathogen, particularly in cystic fibrosis (CF) patients. We have employed transposon mutagenesis to target candidate virulence genes in this organism. In view of the intrinsic resistance of *B. cepacia* clinical isolates to many antibiotics we constructed a mini-Tn5 derivative specifying resistance to trimethoprim. This transposon, together with the previously described mini-Tn5 Cm, were then used to isolate mutants deficient in the production of certain exoproducts. Using mini-Tn5 T_p mutants of the *B.cepacia* CF isolate 715j were obtained which fail to produce the green-yellow fluorescent siderophore pyochelin. Two such mutants were independently obtained and were shown by Southern hybridisation to have single insertions within the same locus. Two mutants exhibiting impaired protease production were isolated following mutagenesis with mini-Tn5 Cm. Southern hybridisations again showed that both contain single insertions at the same locus. Attempts to clone the wild-type genes targeted by these transposons through complementation of the mutants with a genomic library have failed. Therefore, we have cloned the mini-Tn5 Cm and mini-Tn5 T_p transposons, together with flanking DNA sequences, from the mutant genomes into the *E.coli* plasmid pHG165 for DNA sequence analysis. We shall present the results of this analysis.

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Ochrobactrum anthropi is a gram-negative bacillus that has been isolated from activated sludge. In fact, it is able to grow on atrazine by utilizing it as the only source of carbon. One of the enzyme involved in detoxification of xenobiotic is the glutathione S-transferase (GST). We have purified the GST from *Ochrobactrum anthropi* (Oa-GST) and its N-terminal sequence was determined. The apparent molecular weight, of 24 kDa of the protein, was determined by SDS gel electrophoresis analysis. The amino acid sequence obtained showed homology with the known bacterial GST ranging 68-60 %. Immunoblotting experiments performed with antisera raised against Oa-GST, did not show cross-reactivity with other bacterial GST; this reinforcing the concept that bacterial GST are immunologically distinct. The Oa-GST gene has been cloned, the deduced N-terminal sequence was consistent with the sequence of the purified protein. The calculated molecular weight of the encoded protein was 21.7 kD, which was compatible with the relative molecular mass (24 kD) of the purified protein estimated by SDS-PAGE. We hypothesize that Oa-GST could be involved in atrazine dechlorination.

RNA turnover in *Pseudomonas*: identification of genes encoding polynucleotide phosphorylase and a polyadenyl polymerase

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Turnover of mRNA plays an important role in the control of gene expression. Yet, little is known in Bacteria about the mechanisms that control this process.

To study RNA decay in *Pseudomonas* spp. we set out to identify genes that are homologous to *E. coli pnp* (which encodes polynucleotide phosphorylase, PNPase) and *pcnB* (which encodes PAP I, the major polyadenyl polymerase activity found in *E. coli*).

Both genes play an important role in *E. coli* mRNA turnover. Recent studies on *E. coli* mRNA stability have shown that PNPase, together with an RNA helicase, the endonuclease RNase E, and other proteins, is part of the degradosome, a multienzyme complex for mRNA decay. Moreover, it has been shown that polyadenylation of mRNA 3'-end by PAP I is a signal for RNA degradation (1-3).

To clone the *P. putida* homologue of *E. coli pnp* we performed PCR of *P. putida* genomic DNA using degenerated primers designed on two aminoacidic sequences that are conserved in the PNPase of both Gram-positive and Gram-negative bacteria. We thus amplified a 215 nt DNA fragment encoding an orf exhibiting 95% identity with a central aminoacidic sequence of *E. coli* PNPase. This PCR product was used as a probe to clone *P. putida* DNA fragments containing the *pnp* gene.

P. putida PNPase shows 60.3 % identity and 75.8 % similarity with the *E. coli* homologous protein and conserves the RNA binding motifs KH and S1 (4, 5). In *P. putida*, like in *E. coli* and *B. subtilis*, the *pnp* gene is essential for growth at low temperatures and also appears to be unique in *P. putida*, *P. fluorescens* and *P. aeruginosa* genomes.

By a similar approach we identified an *E. coli pcnB* homologue. *P. putida* PAP I shares 46.1 % identity and 66.1 % similarity with *E. coli* PAP I and shows high conservation in the aminoacidic residues specific to the poly(A) polymerase family proteins and the tRNA nucleotidyltransferase. The aspartyl catalytic triad (6, 7) is also conserved.

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Isolation of *Aeromonas hydrophila* in chlorinated drinking water from a Health Care Institution in Argentina

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Aeromonas hydrophila is a pathogen that causes severe infections in humans and many animals. *Aeromonas* species are associated with a wide variety of disease: diarrhea, endocarditis, meningitis and bacteremia specially in young children and healthy individuals. All of these infections are originated from open wounds or by ingestion of these bacteria from food or water sources because they are indigenous to aquatic environments and it can reside in salty or fresh water for long periods. *A. hydrophila* was isolated from chlorinated drinking water from a Health Care Institution. Aliquots of 100 ml of drinking water from all of the pavilions were pass through 0.45µm membrane filter, enriched in Tryptic Soya Broth (TSB) for 24 hours at 37 °C and then isolated onto Cetrimide Agar for 18 to 24 hours at 37 °C. Biochemical identification was done on every no fluorescent colonies with API 20E Test System. *Aeromonas hydrophila* was isolated from drug-addict recovery pavilion samples. This pavilion was the unique where sanitizing program doesn't done for last three years. These findings indicate that a prevention program is necessary in spite of water chlorination.

Acknowledgements are given to Miss. Laura Conca for her technical assistance.

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Forty five isolates of *Pseudomonas aeruginosa* isolated from different infected patients at two hospitals in Valencia were biotyped, serotyped and examined by antimicrobial susceptibility, plasmid content and analysis of chromosomal DNA *EcoRI* ribopatterns (ribotyping). Phenotypic methods showed poor discrimination among strains and plasmids were detected in 16% of isolates. Strains were genetically diverse and 12 different ribotypes of 2 to 7 bands between 5 and 21.5 Kb were defined. All strains shared a common band of 6.0 Kb. Two ribotypes (R1 and R2) were predominant, representing 27% and 38% of all isolates respectively. Ribotypes were not consistently associated with serotypes, but they clearly subtyped strains of the same serotype. This study demonstrated the prevalence of certain strain types associated with infected patients at Valencia hospitals, and confirmed the high typeability and reproducibility of single enzyme ribotyping for epidemiological studies of *Pseudomonas aeruginosa*.

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Monitoring of *Pseudomonas* strains and polycyclic aromatic hydrocarbons (PAHs) concentrations dynamics in model systems containing gray forest soil was used to assess the degradation efficiency by introduced and indigenous microorganisms. The well-known *Pseudomonas putida* G7 [1] and the most active *Pseudomonas* strains BS3701, BS3702 and BS3745 isolated from PAH polluted environments were introduced in model systems at concentration of about one million cells per g of dry soil. Naphthalene and phenanthrene were used as model PAH pollutants. The mathematical model was developed to describe the dynamics of bacteria and PAH and to estimate the kinetic characteristics of the process in soil model systems. The bacteria concentration achieve one million of cells per mg of dry soil in the absence of PAH. But, the maximal introduced bacteria concentrations in model systems with PAHs were up to 2-fold the ones in systems without PAHs. Comparison of introduced microorganisms dynamics in soil model systems with humidity varying from 20% to 70% has showed that systems with humidity level of 40% demonstrate the highest values of economic coefficient and maximal attainable bacteria concentrations. Indigenous microorganisms utilized completely the added naphthalene (2.4 mg per g of dry soil) within 11 days in model soil systems, while naphthalene concentration in sterile soil was about 60% from initial one at the same time due to evaporation. The most effective strain *P. putida* BS3701 utilized naphthalene completely during 3 days. The concentration of added phenanthrene (1 mg per g of dry soil) was not changed in sterile soil model systems during 37 days. The most effective introduced strain *P. putida* BS3702 utilized phenanthrene completely during 6 days. The obtained data suggest that introduced microorganisms can accelerate the PAH remediation in model soil systems.

Identification and genetic analysis of Tn5542, a transposable element carrying the *bedD* and *bedC1C2B4* genes in *Pseudomonas putida* ML2

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Catabolic genes have often been known to be tightly clustered into operons and have also been found to be located within or carried on transposons. Genetic rearrangement events involving genetic recombination and transposition occurring in these catabolic gene clusters have allowed for the acquisition of novel catabolic activities and evolution of new catabolic pathways in microorganisms.

Pseudomonas putida ML2 is able to grow on benzene as its sole carbon and energy source. It possesses two structural genes, *bedD* and *bedC1C2B4*, encoding a NAD⁺-dependent dehydrogenase and benzene dioxygenase, respectively, that are involved in the initial steps of benzene metabolism. Two directly repeated sequences of the IS elements IS1489v1 and IS1489v2 are found to flank the *bedC1C2B4* gene cluster on the catabolic plasmid pHMT112 in *P. putida* ML2, forming a composite transposon with is designated Tn5542. Both IS1489v1 and IS1489v2 contain an identical 1371 bp open reading frame, *tnpA*, that is preceded by a possible ribosome binding site. The *tnpA* gene of IS1489v1 is bounded by a pair of 40-bp imperfect inverted repeats while that of IS1489v2 is flanked only by the left inverted repeat. The *tnpA* gene codes for a 53 kDa polypeptide of 456 amino acids bearing similarity to transposases of *Pseudomonas* sp. EST1001, *S. aureus*, *B. paraperitussis*, *L. mesenteroides* and *M. smegmatis*. The basic nature of the TnpA protein with a deduced pI of 8.93 is also typical of IS-encoded transposases. The different G+C contents of the *bedD* and *BedC1C2B4* genes and their organisation on a composite transposon suggests that both genes were separately acquired and placed in juxtaposition to one another. The formation of such a hybrid 'upper' pathway operon in *P. putida* ML2 with the help of IS elements would confer upon the bacterium the ability to metabolise benzene.

Furthermore, by Southern hybridisation, a third variant of the insertion element IS1489 was identified in pHMT112F, a larger plasmid from which pHMT112 was derived. IS1489v3 is similar to IS1489v2 in lacking the right inverted repeat, with both *tnpA* genes sharing 99.6% identity. An additional copy of IS1489 was also detected on the chromosome of *P. putida* ML2 containing pHMT112F. It is proposed that homologous recombination between repeated regions of DNA in the genome of *P. putida* ML2 harbouring pHMT112F may have been the likely cause for the loss of these two copies of IS1489 in *P. putida* ML2 containing pHMT112.

The major porin OprF as temperature-sensor in the psychrotrophic bacterium *Pseudomonas fluorescens*

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We investigate the effect of temperature on several physiological properties of *Pseudomonas fluorescens* strain MFO. A temperature (around 17°C) seems critical in that it separates two domains where the effects of the temperature are quite different. We have shown, more precisely, that the permeability of the outer membrane to β -lactams, as mezlocillin or cefsulodin, changes with regards to the temperature (1). In these two domains, the major porin OprF displays channels with a threefold higher size at high temperature (28°C) than at low temperature (8°C). This behaviour is also observed for the OprF from another *P. fluorescens* strain, OE.28.3, isolated from the rhizosphere (2). The study of the structure-function relationship of these proteins has been then investigated. Their molecular weight has been found identical (Mw:32.309 Da) by using MALDI-TOF mass spectrometry. This shows that the primary structure of the porins is not modified regardless of the temperature of culture. In other respects, the trypsin digestion kinetics are very different between porins extracted from cultures at 8°C or those at 28°C. This different reactivity could be explained either by a modification of the tertiary structure, or by a modification of the accessibility of the cleavage sites to trypsin due to the presence of lipopolysaccharide. This constituent which can be tightly associated to the porin may influence this trypsin digestion kinetic and also the channel size of the porin. To investigate this hypothesis, the lipopolysaccharide has been extracted, purified and presents mainly a R form for the MFO strain. Moreover, analysis of its phosphorylation state shows differences depending on the two domains of temperature. This variation of the porin structure will be discussed in relation to their function.

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Pseudomonas sp. U2 was isolated from soil exposed to refinery sludge in the south-eastern region of Venezuela and shown to utilise naphthalene as sole carbon and energy source. Previous studies suggested that the genes involved in this function are carried on a plasmid (1). In order to study the catabolism of naphthalene in this strain, we cloned the genes encoding this activity from the plasmid DNA into pUC18, on three fragments which overlap for approximately 1.5-kb. Analyses of deletions and subclones, by biochemical tests and Southern blot hybridisations, showed the catabolic genes to be organised in the same order as their analogous genes in the archetypal plasmid NAH7. They were designated (in order) as *nahAa*, *Ab*, *Ac*, *Ad*, *B*, *F*, *C*, *E*, *D*. Sequence analysis of 5.7-kb containing the genes encoding naphthalene dioxygenase, showed an additional open reading frame (ORF) between *nahAa* and *nahAb* encoding an ISP β -like protein, the deduced amino acid sequence of which is 95% similar to that of an ORF described in *Burkholderia* sp. strain DNT (2). Because expression of this gene is apparently unnecessary for the naphthalene dioxygenase activity and it is widespread among PAH degrading gene clusters, research is underway to determine its function in strain U2.

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Several reports documenting the isolation of organic solvent resistant bacteria have appeared since Inoue and Horikoshi first reported a *Pseudomonas* strain that could grow under toluene (1). *Pseudomonas putida* KT2442 was unable to form visible colonies on a complex medium overlaid by toluene, however, a small portion of the population (ca 10^{-8} - 10^{-6}) did form colonies under xylene. One of these colonies was chosen as a starter strain, and a toluene tolerant strain *P. putida* TOL has been isolated from this toluene-sensitive strain after liquid cultivation under xylene followed by toluene for a month in each case. About 10 to 80 % of the population could form small but readily visible colonies under toluene within 24 hr at 30 °C.

P. putida TOL constitutively overexpressed (ca 10 % of the total protein judged by the intensity of a Coomassie Brilliant Blue stained gel) a ca. 24 kDa soluble protein. This protein was partially purified by an ammonium sulfate fractionation followed by ion-exchange chromatography. The amino-terminal amino acid sequence of the protein was determined up to 29th residue to be P-I-I-N-S-Q-V-K-P-F-N-A-T-A-Y-H-K-G-E-F-V-Q-V-S-E-A-D-L-K-. A data base search revealed that this protein is most similar to the *Escherichia coli* *ahpC* gene product which is the catalytic component of an alkylhydroperoxide reductase. Overexpression of *ahpF* which is localized downstream of *ahpC* in *E. coli* was not observed in the toluene-tolerant strain.

The *ahpC* gene has been reported as an organic solvent-resistant gene in *E. coli* (2). This result strongly suggests a general role of alkylhydroperoxide reductase in organic solvent resistance.

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Cytochrome c and pyoverdine biogenesis in *Pseudomonas fluorescens* ATCC 17400: a new a dual function for an ABC transporter

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Pyoverdine is a siderophore that is essential for the growth of *Pseudomonas fluorescens* in iron-limiting conditions. Based on the complementation of pyoverdine mutants, the *cytA* gene was cloned from *P. fluorescens* ATCC 17400 as part of the gene cluster *cytA-H*. *cytA* encodes a member of the ABC-transporter membrane proteins. *CytA-G* showed high degrees of similarities to different proteins involved in the biogenesis of cytochrome c in several bacteria. Functional analysis demonstrated that *CytA*, as well as *CytB* and *CytC*, are indeed essential for cytochrome c production in *P. fluorescens* 17400. Mutants constructed by disruption of either genes, were unable to grow under anaerobic conditions due to the lack of cytochrome c production. Only the disruption of *cytA*, resulted as well in the formation of, non-fluorescent pyoverdines with altered isoelectric patterns. Construction of a reporter gene *lacZ* transcription fusion with *cytA* showed that the *cyt* cluster is constitutively expressed in both iron-limiting and iron-rich conditions.

CytA was characterized as an inner membrane protein with six membrane-spanning segments. A topological model for *CytA* was constructed based on the analysis of *CytA*-alkaline phosphatase fusions, in which the N- and C-terminal ends were localized in the cytoplasm. All conserved residues in *CytA* and its homologues were localized in three periplasmic loops. Substitutions of the conserved residues by site-directed mutagenesis showed that some residues are essential for cytochrome c, while others are critical for pyoverdine biogenesis only.

Development of “*in vivo* expression technology” (IVET) for the isolation and characterisation of *Pseudomonas fluorescens* genes induced specifically in response to rhizosphere signals .

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Fluorescent pseudomonads are prodigious colonisers of the plant rhizosphere and interactions between pseudomonad and plant can markedly affect plant growth. Certain isolates of *Pseudomonas fluorescens* are capable of promoting plant growth by suppressing the growth of fungal pathogens and this makes them ideal biological control candidates. Unfortunately, their success under field conditions has been limited due to a lack of understanding concerning rhizosphere influences on *P. fluorescens* gene expression. In order to obtain a better understanding of the effect of rhizosphere signals on *P. fluorescens* gene expression, two “IVET” (*in vivo* expression technology) strategies for *P. fluorescens* have been developed. IVET enables the isolation and subsequent characterisation of *in vivo*-induced genes through their ability to drive the expression of an otherwise promoterless gene which is essential for survival *in vivo*. Here we report the construction of an IVET strategy based on a *dapB* mutant of *P. fluorescens*. DAP (Diaminopimelic acid) is found only in the bacterial cell wall and is severely limiting in the rhizosphere, consequently *P. fluorescens* Δ *dapB* is unable to colonise plant roots. The *P. fluorescens* *dapB* gene has been cloned and sequenced, and a promoterless copy incorporated into the IVET system. The IVET vector (IVET-*dap*) has been used to isolate rhizosphere induced (*rhi*) genes of *P. fluorescens* which show elevated levels of expression in the plant rhizosphere.

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Combustion of fossil fuels leads to the release of toxic sulfur dioxides into the environment, contributing significantly to air pollution and being the principal cause of acid rain. Most of the sulfur in petroleum is in the form of heterocyclic organic compounds, which are recalcitrant to the current chemical method for sulfur removal (hydrosulfurization). Dibenzothiophene (DBT) is generally considered as the model compound for sulfur heterocycles present in hydrosulfurization-treated fuel. *Rhodococcus erythropolis* IGTS8 has been shown to remove sulfur from DBT via a sulfur-specific pathway cleaving selectively sulfur from DBT without ring destruction. Three enzymes, DszABC, are responsible for the transformation of DBT into 2-hydroxybiphenyl and sulfite, the last being used by the bacterium as the sole sulfur source (1). The *dszABC* gene cluster has been cloned and sequenced, and its expression appears to be strongly repressed by sulfate and sulfur-containing amino acids (2).

Up to now, only the wild-type strain *R. erythropolis* IGTS8 is being used to develop a commercial biodesulfurization process. However, the design of well-suited recombinant biocatalysts endowed with a desulfurization phenotype could offer new alternatives to develop a commercially viable desulfurization process. Properties such as high tolerance to organic solvents, resistance to heavy metals present in fossil fuels, and the facilities for genetic manipulations, make *Pseudomonas* strains interesting candidates for engineering new biocatalysts for biodesulfurization. Thus, in this work we have constructed two *Pseudomonas* strains, *P. putida* EGSOX and *P. aeruginosa* EGSOX, that efficiently express the *dsz* cluster as a DNA cassette stably inserted into the host chromosome. The engineered cluster is under the control of heterologous regulatory signals and therefore, lacking the native sulfur repression. Moreover, *P. aeruginosa* EGSOX combines two traits of industrial interest, that is, an efficient desulfurization phenotype and the ability to produce rhamnolipid biosurfactants that could help to increase the aqueous concentrations of hydrophobic compounds resulting in higher mass transfer rates in two-liquid-phase bioreactors.

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Bacterial blight of pea is caused by *Pseudomonas syringae* pv. *pisi* and the symptoms are distinct from those of brown spot disease of pea caused by *P. syringae* pv. *syringae*. Strains of *P. syringae* pv. *pisi* exhibit a well-defined race structure based on host cultivar-specificity, whereas some strains of *P. syringae* pv. *syringae* are compatible with all pea cultivars. We have used this commonality of host compatibility to seek avirulence genes, which match resistance genes in pea, from *P. syringae* pv. *pisi*. A cosmid clone from a gene library of the *P. syringae* pv. *pisi* race 4A strain 895A was found to confer a cultivar-specific avirulence on *P. syringae* pv. *syringae* strain 1212R toward pea cultivars carrying the *R3* resistance gene. This avirulence specificity is not observed in the parent strain 895A, which is incompatible only with *R4*-bearing cultivars, nor was the phenotype expressed after transfer of the cosmid to races of *P. syringae* pv. *pisi* tested. The gene has been sequenced, comprising an ORF of 803 bp and is unrelated to the avirulence gene *avrPpiB1* from *P. syringae* pv. *pisi* race 3, which also specifies an *R3*-matching phenotype. The novel gene was shown to have two features typical of *avr* genes - namely low (53%) GC ratio relative to that for *P. syringae* pv. *pisi* and a probable upstream harp box motif: no homology to other sequences in the databases was detected.

Population Dynamics of Fluorescent *Pseudomonads* in the Rhizosphere

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Attempts to use fluorescent pseudomonads to improve crop yield have enormous potential, but to date they have had only limited success in the field. This failure is due in part to the lack of understanding of their population dynamics in the environment, even though this information may be key to their biotechnological exploitation. Diversity among a population of fluorescent pseudomonads isolated from the rhizospheres of wheat and sugar beet seedlings grown in soil taken from the field, has been investigated by ribotyping. From a sample of 450 isolates, 177 distinct ribotypes were detected but only two ribotypes were isolated from both plant types, and the bulk soil, and only one, the most common ribotype (ribotype A), was isolated from all samples. In a subsequent study carried out seven months later, diversity was assessed during early seedling growth using soil taken from the same field site. On each sampling occasion, more ribotypes were isolated, showing the extent of the diversity within the population, but the most common ribotypes identified in the first study were still present in the soil after seven months, and predominated in the rhizosphere during the first 23 days of seedling growth. Tests were carried out to ascertain which phenotypes, if any, were associated specifically with the persistent ribotypes. When metabolic profiles were compared, the persistent ribotypes were found to be distinct from those that were only isolated once. The ability to utilise certain organic acids appears to be an important trait in rhizosphere competent strains.

Ribotype A was genetically marked using a mini Tn-5 cassette, and introduced back into the rhizosphere alone, and in competition with either a marked ribotype detected only once in soil (ribotype X), or one detected only once in the rhizosphere (ribotype Y). Preliminary results show that ribotype A colonised the rhizosphere well when introduced on its own, compared to X and Y that were less able to colonise. However, the proportion of A in the rhizosphere was enhanced in the presence of either X or Y. The proportions of X and Y were also significantly increased in the presence of A, indicating cooperative interactions between the introduced strains.

Isolation and characterization of the *Pseudomonas putida* DOT-T1 which grows and metabolizes toluene and other aromatic hydrocarbons at high concentrations.

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Aromatic hydrocarbons are extremely toxic for living organisms. They are preferentially partitioned in cell membranes, where they disrupt membrane structure. As the solvent accumulates, the membrane loses its integrity and becomes more permeable to protons and ions, which leads to cell death.

A *Pseudomonas putida* strain able to grow in medium containing up to 90% (v/v) toluene was isolated. The catabolic potential of the isolate was expanded for a wider range of other aromatic compounds.

The mechanisms responsible for the resistance to solvents in this isolate have been partially elucidated. Modifications in lipid membrane composition appear to play an important role by increasing cell membrane rigidity. A key process is the rapid transformation of the fatty acid *cis* 9,10-methylen hexadecanoic acid to *cis*-9-hexadecenoic acid and the subsequent transformation of the latter into the *trans* isomer. Adaptations in the phospholipid headgroups have also been observed, with an increase in the level of cardiolipin (diphosphatidylglycerol) and a decrease in the level of phosphatidylethanolamine.

Studies with ¹⁴C-toluene and ¹⁴C-trichlorobenzene found that the aromatic compound is exported from the membrane in an energy-dependent process. The synthesis of the efflux system is constitutive thus appears that solvent-resistant strains always exhibit a mechanism that prevents the accumulation of solvents in the cytoplasmic membrane. A catabolic pathway for toluene metabolism has been proposed. Tn5 mutants unable to grow in the presence of toluene were isolated and characterized to elucidate the route of and the genetic components, and key genes have been cloned.

The ability of *Pseudomonas putida* cells to tolerate high concentrations of organic solvents makes them particularly suitable for bioremediation purposes. We report the ability of different toluene-degrading strains to establish in soils that contained high levels of solvents. These strains successfully eliminated toluene from these highly contaminated soils.

Comparative AP-PCR typing of clinical and environmental isolates of multidrug-resistant *Pseudomonas aeruginosa* strains

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The family *Pseudomonadaceae* is often responsible for opportunistic infections in debilitated patients, and *Pseudomonas aeruginosa* is the species most frequently isolated from clinical specimens. Now it seems that *Pseudomonas aeruginosa* may emerge as an important pathogen responsible for infection diseases in children.

In this study we have evaluated several colonizing and infecting, multidrug resistant *Pseudomonas aeruginosa* strains. 80 isolates of *Pseudomonas aeruginosa* were collected from 16 patients, 15 children plus one adult, randomly selected from a test group of 100 cases presented during twelve months in the years 1995-1996, in a pediatric laboratory at the city of Cartagena, Colombia.

All the isolated strains from diarrhea, cystic-fibrosis, pneumonia and urinary tract infections were identified as *Pseudomonas aeruginosa* by API. All of them show similar colony morphology and minor but distinct susceptibility differences.

The genomic typing of these isolates by arbitrarily primed-polymerase chain reaction (AP-PCR) show an striking similarity among isolates from the same or different kind of infection. Based upon this data, we should conclude that some patients were infected by similar if not identical strains.

Identification of different species of *Pseudomonas* by genomic PCR fingerprint using species-specific but strain-independent patterns

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The high genomic variability among different strains of *Pseudomonas aeruginosa* and other related species has been reported in many studies, using arbitrarily primed PCR and other genomic fingerprinting methods, and this variability is of wide utility for the identification of bacterial strains in epidemiological studies.

But PCR-based genomic fingerprinting would also be a very convenient taxonomic tool for differentiating species. The requirement for this application is to identify genomic sequences both polymorphic within the genus but highly conserved within every species. A direct identification of such genomic sequences would be difficult because of the large cloning and sequence analysis project that it would involve. Having in mind the enormous power of the genomic fingerprinting approach by arbitrarily primed PCR, we carried out a systematic search to identify oligonucleotide sequences that generate patterns of PCR amplified bands with the characteristics indicated above, that is, polymorphic among different species but non-polymorphic between different strains of the same species.

The preliminary results of our search using different strains of *Pseudomonas aeruginosa*, *P. fluorescens*, *P. cepacia*, *P. stutzeri*, *P. corrugata*, *P. psyringae*, *Acinetobacter lowfy*, *A. calcoaceticus*, *Alcaligenes faecalis* and other bacterial genus and species, indicate that specific oligonucleotide sequences can be found which fulfill the requirements for taxonomic application.

Physiological and genetic characterisation of the PAH degrading *Pseudomonas alcaligenes* strain PA-10.

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Pseudomonas alcaligenes strain PA10, an environmental isolate was found to be capable of degrading the three low molecular weight polycyclic aromatic hydrocarbons (PAH), phenanthrene (10ug/ml), anthracene (2.5ug/ml) and fluorene (10ug/ml); when these PAH were supplied as the sole carbon sources in the growth medium. Complete mineralisation of phenanthrene was observed with less than 1% of the initial PAH remaining after 4 days of growth. Phenanthrene is degraded via the well established pathway involving 1-Hydroxy-2-naphthoic acid, salicylate and catechol (1). Fluorene degradation proceeds at a slower rate with a 90% reduction in the initial fluorene concentration being observed following 20 days of growth. HPLC analysis shows fluorene degradation resulting in the formation of 9-fluorenone and 9-fluorenone in the growth medium. This is similar to a fluorene degradation pathway reported for an *Arthrobacter* sp. strain F101 (2). When anthracene was supplied as the sole carbon source significantly lower degradation levels were observed with almost 80% of the initial PAH compound still remaining after 20 days of incubation, when compared to an abiotic control. 2-Hydroxy-3-naphthoic acid and salicylate were detected in the growth medium suggesting that *P. alcaligenes* degrades anthracene via the well studied pathway involving 1,2-Dihydroxyanthracene as the key intermediate (3).

A number of genes encoding dioxygenases responsible for bacterial naphthalene catabolism have been cloned and sequenced (4,5). Many regions of these genes are highly conserved (> 90% homology), between *Pseudomonas* species. PCR primers designed from one of these consensus regions were used to amplify a 540bp product, using plasmid DNA isolated from strain PA10 as template. This PCR product is now being used together with an inverse PCR approach to genetically characterise this 76kb plasmid. Further analysis will be shown.

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Characterization of MexC-MexD-OprJ Efflux System in *mexA-mexB-oprM* Region-Deleted Mutants of *Pseudomonas aeruginosa*

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Expression of the multidrug efflux system MexC-MexD-OprJ in the *nfxB* mutant of *Pseudomonas aeruginosa* has been characterized as contribution to resistance against fluoroquinolones and fourth generation cepheims (cefpirome and ceftazidime), but not to β -lactams including the ordinary cepheims (ceftazidime and cefoperazone) (1, 2, 3). These *nfxB* mutants also express the second multidrug efflux system, MexA-MexB-OprM, due to incomplete transcriptional repression of this second operon by the *mexR* gene product. To characterize exactly contribution of the MexC-MexD-OprJ system to alteration of the susceptibility in *P. aeruginosa*, a site-specific deletion system of the bacterial chromosomes was employed to remove the *mexA-mexB-oprM* region from the wild-type and *nfxB* strains of *P. aeruginosa*. Use of the *nfxB* mutants lacking the *mexA-mexB-oprM* region clearly indicated that the MexC-MexD-OprJ efflux system was involved in resistance to fluoroquinolones, the fourth generation cepheims, and the ordinary cepheims but not to carbenicillin or aztreonam. The murine monoclonal antibodies and rabbit polyclonal antisera against each component of the two efflux systems were prepared. The immunoblot assay using these antibodies revealed the reduced production of the MexA-MexB-OprM components in the ordinary *nfxB* mutants. This reduction of these components explains the hypersusceptibility to β -lactams in the ordinary *nfxB* mutants.

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Sequence Analysis of the TOL Plasmid pWW0 from *Pseudomonas putida*

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pWW0, "the archetype of the family of TOL plasmids" isolated from *Pseudomonas putida* mt-2, was the first of the TOL plasmids identified. Its size is estimated at 117kb and restriction maps for *EcoRI*, *HindIII*, *SacI*, *XbaI* and *SfiI* have been constructed. The catabolic gene cluster encoding the biodegradation of toluene and xylenes and their oxidation products, has undergone extensive analysis. Research has been carried out to identify the catabolic genes, their regulation and the pathways in which they are involved. Nucleotide sequence exists for all the *xyl* regulatory and structural genes.

Although pWW0 is known to belong to the IncP-9 group of plasmids and is capable of transfer to *E. coli*, little is understood about what exists in the non-catabolic region of the plasmid. Transposon mutagenesis led to the estimated locations of the replication and transfer functions, but the associated genes have not yet been isolated. The backbone of pWW0 (outwith the catabolic region) must include replication and transfer functions, but the nature of the remaining genes is unknown. Studies demonstrating homology between the backbones of a variety of degradative plasmids suggest that a core group of self-transmissible replicons exist in the environment as vehicles for the spread of various catabolic modules.

Recent findings have shown the similarity between Pseudomonads isolated from both clinical and environmental contexts. *Pseudomonas aeruginosa* can cause opportunistic infections and specific diseases such as cystic fibrosis whereas *Pseudomonas fluorescens* is capable of polyketide antibiotic production and is a potential source of antibiotic resistance genes. Some strains of *P. aeruginosa* appear to have the potential for pathogenesis in both plants and animals. Plasmids could potentially provide genes required for adaptation to these specific habitats and may be important in the spread of such genes. Indeed other IncP-9 plasmids have been identified on the basis of their antibiotic resistance phenotype rather than their degradative functions.

To understand the nature of this group of vectors for gene flow between Pseudomonads we are sequencing the backbone of pWW0 to complete the nucleotide sequence of this plasmid. To date two insertion elements have been identified and some of the sequence of genes involved in conjugal transfer. An update will be given of the sequencing progress.

Genetic relationships among genomovars and strains of *Pseudomonas stutzeri* based on rapid molecular typing methods

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Eight genomic groups in *Pseudomonas stutzeri* (called genomovars, genomic groups without taxonomic status) have been well characterized by DNA-DNA similarity analysis, 16S rRNA gene-sequencing, and other high resolution methods (total protein patterns, macrorestriction fragment analysis, physical genomic map). Although the results have been consistent with the genomovar subdivisions observed, all these methods are complicate and time consuming. In this study we have developed rapid molecular methods for the identification of the species and other molecular typing methods for the genomovars and strains characterization.

The identification of *P. stutzeri* was easily accomplished using the combined results of two tests based on PCR, using specific primers based on the 16S rDNA sequence for *P. stutzeri*, following a RFLP analysis by treatment of the 16S rDNA PCR products with the hexameric site-specific restriction endonuclease *Bam*HI.

For the intraspecific characterization of the strains, restriction patterns of the 16S rDNA genes were determined using five different enzymes, that allowed the identification of some bands specific for genomovars.

We have also used rapid molecular typing methods based on PCR amplification with oligonucleotide primers related to consensus sequences derived from highly conserved palindromic inverted repeat regions found in enteric bacteria (REP) and enterobacterial repetitive intergenic consensus sequences (ERIC). Furthermore, we have applied an identification method based on the PCR amplification of the 16S-23S rDNA intergenic spacer region (ISR) that have been also analyzed by single strand conformation polymorphism (SSCP).

The above-described methods have been applied to a collection of 14 representative strains of *P. stutzeri* and its potential application has been evaluated for rapid and correct discrimination among members of the species *P. stutzeri*. At the same time, a clear separation from the 14 type strains of the true pseudomonads studied was achieved. These molecular typing methods are useful tools for the detection, monitoring and discrimination of new *P. stutzeri* isolates from clinical and environmental samples.

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Changes in cell size and morphology during and after multiple-nutrient starvation

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The morphological variations of a strain of *Ps.aeruginosa* were analyzed under different conditions of nutrient starvation and/or stresses, and after restoring the normal conditions. Under conditions of carbon starvation, the strain *Ps.aeruginosa* entered a stationary growth phase, the regenerative capacity remained unaltered with colonies of normal size and morphology.

After 48 h of starvation, we observed spherical shaped cells with reduced dimensions, which reached a minimum volume (about 0.33 nm) at about the eleventh day. Reactivation experiments showed an increment of size 20 minutes after addition of carbon source and normal size after 90 minutes. However, increase in cellular mass was observed after 18h, while an increment in oxidative metabolism after 3,40h and a successive peak after 5h.

Under phosphate starvation, we observed a progressive increase in mass, while the regenerative capacity was reduced. After the fourth day of starvation, colonies of reduced dimension appeared, while the cells maintained a rod aspect, even if with a reduced volume, and spherical forms were sporadically seen.

Reactivation experiments showed an increment of size 20' after addition of phosphate source and normal size after 120'. However, increase in cellular mass was observed after 24h, while an increment in oxidative metabolism appeared after 3,20h, but the successive peak was longer to carbon.

Under both phosphate and carbon starvation, there was a stationary phase while the regenerative capacity was constant, with a small quantity (10%) of reduced dimension colonies with irregular outlines; the cells assumed a spherical morphology with reduced volume, as observed in carbon starvation.

When *Ps. aeruginosa* is exposed to 45°C there was the formation of small rod cells. Reduction of cellular size was also observed at 4°C.

Similar analyses of carbon, phosphate and both carbon and phosphate starvation were performed at 45°C or at 4°C. Morphological analyses showed a significative difference in phosphate starved cells at 45°C where small strepto-bacillar cells with irregular divisions sets and spherical cells were seen. On the other hand at 4°C cells retained a small rod-shape. In reactivation experiments cells showed an increment of size between 20 minutes and 120 minutes. However, increase in cellular mass was observed after 24h for carbon starved cells at 45°C or 4°C, while an increment in oxidative metabolism after 30' in cell at 4°C was observed.

The data show a different morphological and functional adaptation under nutrient and multiple-nutrient starvation and/or stresses, seen through the lack of: 1) a stationary phase, 2) a spherical morphology assumed by the cells in conditions of starvation e.g. for carbon, 3) the appearance of phenotypic variants, 4) the variations of oxidative metabolism; all these pictures are related to the survival, the growth and regenerative capacity of the culture.

Growth, regenerative capacity and variations of the oxidative metabolism in a strain of *Ps.aeruginosa* under simultaneous-multiple-nutrient starvation and environmental stresses.

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The growth, regenerative capacity and variations of the oxidative metabolism in *Ps.aeruginosa* (ATTC 27853), were analyzed under simultaneous-multiple-nutrient starvation (carbon, phosphate and nitrogen) and environmental stresses (O_2 , temperature of 4°C and 45°C) for a period of 15 days.

Under conditions of carbon starvation, the *Ps.aeruginosa* strain enters a stationary growth phase, but the regenerative capacity is unaltered. The respiratory activity, evaluated using MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) on solubilized cells, after a decreasing phase, stays constant during all the observation period.

Under phosphate starvation, we observed a progressive increase in mass, while the regenerative capacity was reduced, with periodic oscillations. The respiratory activity had a cyclic trend.

We could not detect any growth at 4°C or at 45°C; respiratory activity was similar in both conditions, while the regenerative capacity was reduced at 45°C.

In nitrogen starvation no growth, but a very high respiratory activity was observed. The regenerative capacity was constant for all 15 days.

Under both phosphate and carbon starvation, there was an immediate decline in cellular mass, while the regenerative capacity was constant. The respiratory activity was almost constant for all the period of observation, with values significantly higher than in absence of only carbon or only phosphate.

When the cultures were shifted in carbon-free medium and incubated at 4°C or at 45°C, an immediate decline in cellular mass, in respiratory activity and in regenerative capacity was observed. In contrast, when experimentes were performed in phosphate-free medium at 4°C or at 45°C, an increase in cellular mass was observed, while respiratory activity and regenerative capacity were dramatically reduced.

The data show a different functional adaptation of *Ps. aeruginosa* in simultaneous -multiple-nutrient starvation or stress, compared to that observed in absence of only one nutrient or when the stress was applied in already starved cells.

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- 4) J. Bacteriology 174 (1994) 7-14

POSTERS ABSTRACTS

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In *P. aeruginosa* gluconate can be transported into the cell then phosphorylated, or it can be oxidized to 2-ketogluconate in the periplasm for independent transport and phosphorylation. In order to study these two pathways we first isolated mutants blocked in the ability to utilize 2-ketogluconate (*kgu-11*). Starting with *kgu-11* we then obtained mutants blocked in the transport (*gnuT*) and phosphorylation (*gnuK*) of gluconate. Mutants with deficiencies in either pathway (either *kgu* or *gnu*) grow normally on gluconate indicating that both pathways are physiologically relevant. These mutations map to 49 min. on the PAO chromosome with *gnuT* and *gnuK* being particularly tightly linked. We cloned both the gluconokinase (*gnuK*) and the gluconate permease (*gnuT*) on a complementing cosmid. Both activities retained their inducible phenotype. *gnuK* and *gnuT* were subsequently subcloned together on a 3 kb *Sall* DNA fragment as well as individually on smaller plasmids, suggesting that both *gnuK* and *gnuT* are independently expressed. DNA sequence analysis of the 3 kb *Sall* fragment indicates a relative gene order of *gnuK* followed by *gnuT*. The open reading frames encode proteins of 18 and 48 kd respectively, and are separated by 96 bp. This DNA fragment also contains a gluconate regulatory protein (*gnuR*) divergently expressed from *gnuK*. The deduced amino acid sequences of the *P. aeruginosa* gluconate regulator, gluconokinase and gluconate permease are 37-40% identical to respective *Escherichia coli* and *Bacillus* homologs.

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In *P. aeruginosa* succinate or other TCA cycle intermediates repress the synthesis of multiple catabolic operons. Mutants affected in this catabolite repression were previously isolated and used to clone the *crc* gene (2,4). Unexpectedly the *crc* gene product showed homology to the family of AP-endonucleases, a group of related proteins that share multiple nuclease activities. However, we were unable to show any AP-endonuclease activity (either *in vitro* or *in vivo*) or any other nuclease activity (1). Here we have modeled a structure for the Crc protein based on the crystal structure of *Escherichia coli* Exonuclease III (3) and we observe a general conservation of structure, particularly for residues involved in catalysis by Exonuclease III. We hypothesized that Crc has a nuclease activity perhaps on an unidentified substrate or with an unknown cofactor. To test this hypothesis we have screened our *crc* mutants by Western blot for ones that make full length protein, used PCR to amplify, then sequenced two containing single amino acid substitutions. One mutation changes Serine 148 to Leucine. By analogy to ExoIII, Serine 148 should make a hydrogen bond that stabilizes the transition state for phosphodiester cleavage. The mutation to leucine would remove the hydrogen bonding potential and introduce a large residue into the active site. Hence this mutant is consistent with a nuclease activity for the Crc protein. The other mutation changes Arginine 229 to Cysteine. This should affect a residue on the surface opposite the active site. We suggest that this either affects the overall structure of the protein or interferes with the regulation of the activity of the protein. To directly test the hypothesis, site-directed mutagenesis was used to change Aspartic acid 35 in Crc to Alanine (D35A). In Exonuclease III this carboxyl side chain coordinates the essential Mg²⁺ ion. The D35A protein complements *crc*, hence *crc* function does not include a nuclease activity that is important for catabolite repression.

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Burkholderia cepacia is an important opportunistic pathogen that can lead to rapid deterioration or death of CF patients. Flagella, consisting of flagellin filament, hook and basal body have been implicated as invasive virulence factors in a number of bacteria. In addition, the bacterial flagellin gene is highly variable in sequence, a property that can be exploited for specific detection / identification, or for studying genetic variation within populations. A comparison of flagellin protein sizes revealed distinct groupings of *B. cepacia* strains. Flagellin genes were cloned and sequenced from two strains of *B. cepacia* representing different flagellin groups, and from one strain of *Burkholderia pseudomallei*, the causative agent of melioidosis. The sequence information was used to design N-terminal and C-terminal oligonucleotide primers for PCR amplification of *B. cepacia* flagellin genes. PCR amplification, in combination with RFLP analysis was used to study flagellar variation amongst isolates of *B. cepacia*. In a study of 17 isolates, 10 different flagellar types could be identified by using the restriction enzymes *Hae*III and *Msp*I. EM analysis was used to compare flagellar width between strains.

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The lipopeptide toxin tolaasin is the primary pathogenicity determinant of *Pseudomonas tolaasii* in causing brown blotch disease of the cultivated mushroom (1). Tn5 mutagenesis of the wild type pathogenic strain *P. tolaasii* 1116S and analysis of tolaasin deficient mutants resulted in identification of the tolaasin gene cluster (2). Since three 435 - 465 kD proteins are encoded by this cluster, it was deduced that tolaasin is synthesised non-ribosomally by peptide synthetases. In order to facilitate elucidation of the molecular basis of tolaasin biosynthesis, cloning and sequencing of the tolaasin gene cluster was performed. The tolaasin gene cluster was isolated by cloning the DNA flanking *tox::Tn5* insertions followed by constructing genomic libraries of the Tn5 tagged tolaasin negative mutants. 31 cosmid clones were isolated from eight different Tn5 insertion sites. Physical mapping of these clones showed that they overlapped and cover the entire 65 kb cluster. DNA sequencing of these clones was carried out in collaboration with the Human Genome Mapping Project Resource Centre Cambridge UK by using the shot-gun strategy. Analysis of the resulting DNA sequence has showed that the tolaasin synthetases contain domains with high homology to those in the pyoverdine, surfactin and antibiotic pristinamycin synthetases. Progress in the sequence analysis of the tolaasin gene cluster will be reported.

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Tn5 tagging of the phenol-degrading gene on the chromosome of *Pseudomonas putida*

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Transposon mutagenesis was performed by the method of conjugal transfer in order to identify and characterize genes encoding enzymes involved in the pathway of phenol utilization. *Escherichia coli* which carries the Tn5-132 was mated with *Pseudomonas putida* SM25 as a host. We selected a mutant SM25-M that could not utilize phenol as a carbon source. Chromosomal integration of the transposon was confirmed by Southern analysis, successfully tagging the gene related to phenol-utilizing pathway. The mutant SM25-M utilized β -ketoadipate and succinate, while did not utilize phenol and catechol. The activities of enzymes, phenol hydroxylase, catechol 1,2-oxygenase, and *cis,cis*-muconate lactonizing enzyme which are involved in *ortho*-pathway were determined. Genetic complementation of the mutant SM25-M tagging Tn5-132 was tested with the various plasmids, pPAN 4-R(*cat*BCDE) and pRSU2(*cat*B). From the results, the mutated gene through transposon insertion was identified as *cat*B gene which encodes a *cis,cis*-muconate lactonizing enzyme.

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The novel genes, *cbbQ* and *cbbO*, located downstream from the RubisCO genes of *Pseudomonas hydrothermophila*, affect the conformational states and activity of RubisCO

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cbbQ, *cbbO*, and *cbbD* genes are located downstream from the RubisCO genes (*cbbLS*) in a genome of a thermophilic hydrogen-oxidizing bacterium, *Pseudomonas hydrothermophila*. *cbbQ* has high similarity to the *nirQ* gene from *P. aeruginosa* or *P. stutzeri*, or *norQ* gene from *Paracoccus denitrificans*. *cbbD* has high similarity to *norD* gene from *P. aeruginosa* or *Pa. denitrificans*. However, no gene which is highly similar to *cbbO* was found in the databanks. Recombinant RubisCO enzymes were purified from *E. coli* cells which were transformed with plasmids expressing *cbbLS*, *cbbLSQ*, *cbbLSO*, or *cbbLSQO*. Co-expression of *cbbQ* and/or *cbbO* with *cbbLS* made the maximal rates of carboxylation (V_{max}) of the recombinant RubisCOs about two-fold higher than that of the enzyme derived from the plasmid possessing only *cbbLS*. The RubisCOs with high V_{max} prepared as mentioned above also had a high stability when undergoing ultrasonic treatment. The results of the circular dichroism spectra and the 8-anilino-1-naphthalenesulfonate binding assay indicated that these recombinant RubisCOs were conformationally different to each other.

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Branched chain keto acid dehydrogenase (BCKAD) is a multienzyme complex involved in the metabolism of valine, leucine, and isoleucine; BCKAD catalyzes the oxidative decarboxylation of branched chain keto acids which have been formed from branched chain amino acids. The four structural genes of *P. putida* BCKAD are encoded by the *bkd* operon, and the positive regulator of the *bkd* operon, BkdR, is encoded upstream of and divergently transcribed from the operon. *P. putida* BCKAD has been shown to be subject to catabolite repression in the presence of carbon sources such as glucose and succinate (1); however, very little is known about the mechanisms involved in catabolite repression control in pseudomonads. The only protein shown to be involved in this control is the product of the *P. aeruginosa* *crc* gene: it has been shown to be responsible for repression of multiple, independently regulated pathways (2). BCKAD activities were determined for wild type *P. aeruginosa* PAO1 and *crc* mutant cultures grown in the presence of various substrates, and relief of repression in the presence of glucose and succinate was seen in the mutant cultures. When the *crc* mutant was complemented with *crc* on a plasmid, repression was no longer relieved in these cultures. Since expression of the *P. putida* *bkd* operon is also controlled by BkdR, β -galactosidase activities were measured of a *P. putida* strain (JS386) containing a chromosomal *bkdR-lacZ* translational fusion grown in the presence of various substrates, and glucose was shown to repress *bkdR* transcription (3). These data implicate *Crc*'s involvement in catabolite repression of the *bkd* operon by repression of *bkdR* transcription.

No DNA-binding ability could be identified for *Crc* using electrophoretic mobility shift assays with the intergenic region between *bkdR* and the *bkd* operon, suggesting that *Crc* interacts with another regulatory protein that binds to this region and either activates or represses *bkdR* transcription. To identify this factor, we are currently attempting transposon mutagenesis of JS386. To isolate the *P. putida* *crc* gene, the *P. aeruginosa* *crc* gene was used to screen a PpG2 chromosomal DNA cosmid library, and a positive clone has been identified and will be sequenced.

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The ability of levan- and alginate-production and the influence of environmental factors on the production of these exopolysaccharides (EPS) was estimated in phytopathogenic and saprophytic members of the *Pseudomonas* group, especially for the *P. syringae*-pathovars. The levansucrase from *P. syringae* pv. *phaseolicola* was purified and its biochemical and physiological properties investigated. Molecular weight and rheological behaviour of levan produced from sucrose by purified levansucrase under different production conditions regarding temperature and osmolarity varied broadly. Under conditions of sucrose-shortage levan was rapidly degraded. Investigated isolates varied in the amount of levansucrase in culture supernatants and in their sensitivity to temperature and nutrient availability. A polyclonal antibody directed against the levansucrase of *P. syringae* pv. *phaseolicola* was raised in rabbits and used to detect levansucrase in culture supernatants and to check the immunological relationship of the enzyme between several species of phytopathogenic bacteria. The antibody detected levansucrases under non-denaturing (ELISA) and denaturing (immunoblot after SDS-PAGE) conditions in all investigated *P. syringae* pvs. and *Erwinia amylovora*. In Southern hybridization experiments with *Pst*I digests from genomic DNA of several *Pseudomonas* species with the *lsc*-gene from *Erwinia amylovora* as a probe presence of the levansucrase gene was confirmed in 15 cases, although enzyme activity was only detected in 11 isolates. Depending on nutrient availability and temperature the amount of levansucrase in culture supernatant varied. Cloning and mutagenesis of the gene for levansucrase in *Pseudomonas syringae* pvs. is in progress. Production of alginate was temperature-dependent. For all isolates a markedly increased amount of alginate was found in higher temperature. The *algD* gene encoding the key enzyme for alginate synthesis has been cloned from *P. syringae* pv. *phaseolicola* and mutagenized by an Ω -insertion. To investigate gene regulation *in vitro* and *in planta* the construction of GUS-mutants is in progress.

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Ralstonia (Pseudomonas) solanacearum is a causal agent of bacterial wilt disease of various plants. This bacterium is known to be very mutable. For example, successive subculturing of the wild type on nutrient-rich medium deprives of its pathogenicity or virulence easily as well as changes of colony morphology (from fluidal to nonfluidal) on the TZC medium. But the mechanism of these mutation has been uncertain so far. We assessed the difference between wild type and mutants which lost pathogenicity by using RAPD analysis. Mutants of *R. solanacearum* MAFF 301520 (race 1/biovar 4) were isolated from the subculture which had been serially cultured over ten times on the TZC medium and they were certified the lost of pathogenicity to tomato. RAPD analysis using one of tested primers (BOX) showed that the wild type differed from its pathogenicity mutants. Two DNA bands (ca. 0.85 kb from wild type and ca. 1.1 kb from the mutant) which differentiated each strain were then recovered and subsequently cloned into PCR-Script cloning vector. The partial sequence analysis of the cloned DNA fragments revealed that these two include highly common conserved region in the both ends. Further analysis is now in progress.

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Pseudomonas sp. are able to bind numerous cell surfaces, animals or plants, to develop infectious diseases or food contamination. This adherence is a complex phenomenon involving several bacterial adhesins such as pili, lipopolysaccharide, exopolysaccharides and outer membrane proteins. Each ligand interacts with specific receptors on the epithelial cell target. We are interested in adherence of fluorescent *Pseudomonas* (*P. aeruginosa*, *P. fluorescens* and *P. putida*) to animal epithelial cells, specially interactions of these bacteria with a protein of the extracellular matrix, fibronectin (Fn), which also presents opsonic activity in plasma.

By *in vitro* experiments, we studied the binding of purified plasmatic fibronectin to these bacteria and tried to characterize outer membrane proteins (OMP) involved in these interactions. First we have shown by specific immunorevelation that Fn bound to bacteria immobilized on nitrocellulose disc. We have also extracted OMPs with a zwitterionic detergent. After separation on SDS-PAGE and electrotransfer on a nitrocellulose sheet, immobilized proteins were incubated with plasmatic fibronectin before revelation of the complexes with anti-fibronectin polyclonal antibodies and a phosphatase alkaline-conjugate.

Electrophoretic profiles and Fn binding responses showed differences in lightened OMPs between exponentially growth and stationary phase for each strain used. A majority fibronectin binding-protein was revealed at 48 kDa, 60 kDa and 50 kDa for stationary *P. aeruginosa*, *P. fluorescens* and *P. putida* respectively. With exponentially growing bacteria, additional proteins were lightened by Fn binding: 68 and 110 kDa for *P. aeruginosa*, 45 kDa for *P. fluorescens*, 60 and 70 kDa for *P. putida*. All proteins that bound plasmatic fibronectin corresponded to thick bands on SDS-PAGE and should be majority expressed OMPs from each stage of growth. Identification of the region of the molecule involved in such a binding is in progress : either fibronectin N-terminal segment which is known to bind *Staphylococcus aureus* or C-terminal region containing the RGD sequence needed for cell interaction.

Reactivation of catechol 2,3-dioxygenase (XylE) by a novel [2Fe-2S]ferredoxin (XylT) of *Pseudomonas putida* mt-2

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The TOL operon from *Pseudomonas putida* carries a set of genes responsible for the complete degradation of alkylbenzenes. The catechol 2,3-dioxygenase, encoded for *xylE*, catalyzes the opening of the aromatic ring, a key step in this degradation pathway. The enzyme undergoes oxidative inactivation during the catalytic cycle, especially with alternative substrates like 4-methylcatechol. In a previous study, the *xylT* gene product has been proposed to reactivate the catechol 2,3-dioxygenase *in vivo* (1). In the present work, the *xylT* gene was overexpressed in *E. coli* and its product was purified. It was characterized as a [2Fe-2S]ferredoxin that displays unique molecular properties. It was also demonstrated that purified XylT reactivates XylE *in vitro* in a reaction which is concentration-dependent and XylT-specific. The XylT reactivation was correlated with a reduction of the iron atom present at the catalytic site of the enzyme.

The molecular interaction between XylT and XylE, studied *in vitro* by cross-linking experiments using a carbodiimide, gave a major cross-linked product which was identified as resulting from the covalent binding between XylT and one XylE subunit. A peptidic analysis of this product has been undertaken.

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Cloning and sequencing of steroid and aromatic hydrocarbon catabolizing enzymes in *Comamonas testosteroni*

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Steroids and aromatic hydrocarbons (AHs) are growth substrates for a great variety of microorganisms, including strains of the genus *Comamonas*. Complete assimilation of these substrates to the final production of H₂O and CO₂ is achieved through complex metabolic pathways which comprise several inducible proteins.

In previous investigations we have identified several proteins specifically expressed in the presence of the steroid testosterone in *Comamonas testosteroni* by using two dimensional gel electrophoresis. Eleven proteins induced and one protein repressed by testosterone in the growth medium were identified and their N-terminal amino acid sequence determined. Identified proteins indicate that testosterone simultaneously induced the expression of steroid and aromatic hydrocarbon catabolizing enzymes in *C. testosteroni*. In the present study we successfully performed the molecular cloning, sequencing and structural analysis of the respective encoding genes. According to the high abundance of steroids in nature it is possible that steroids represent the original substrate of many of these enzymes, and that steroids may be involved in the regulation of these inducible proteins. Since only little information has been available on the steroid catabolic pathway and its regulation, based on our results we will now be able to shed some light on the regulatory network involved in steroid metabolism in *C. testosteroni*.

Proton transfer in benzyl alcohol dehydrogenase during catalysis: alternate proton-relay routes

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His51 in horse liver alcohol dehydrogenase (ADH_E), has been proposed to act as a proton donor/acceptor in the NAD⁺/NADH-dependent oxidation/reduction of alcohol/aldehyde. The residue corresponding to His51 of ADH_E is Val45 in benzyl alcohol dehydrogenase (BADH) encoded by TOL plasmid pWW6. The 3-D structure of BADH modeled from the crystal structure of ADH_E suggests that His41 of BADH corresponding to Arg47 in ADH_E would play the role of His51 in ADH_E. To test this hypothesis, mutants of BADH, in which His41 was replaced by Gln (His41Gln) and/or Val45 was replaced by His (Val45His), were constructed. The kcat/Km value of the His41Gln mutant for benzyl alcohol was 125-fold lower than that of wild-type BADH, while the kcat/Km value of the His41Gln/Val45His double mutant was 12-fold higher than that of the His45Gln mutant. The kcat/Km value of the His41Gln mutant increased with increasing concentration of exogenous amines. These results suggest that His45 in wild-type BADH, exogenous amines in the His41Gln mutant, and His45 in the double mutant act as a general base catalyst during alcohol oxidation.

Effect of solvent adaptation on the resistance to antibiotics in the solvent-resistant *Pseudomonas putida* S12

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Organic solvents like toluene are toxic for organisms because they accumulate in and disrupt cell membranes¹. However, in the recent years exceptional strains have been isolated which can grow in the presence of a second phase of organic solvents earlier believed to be lethal. To uncover the mechanisms behind this remarkable solvent-tolerance we started to study the solvent-tolerant strain *Pseudomonas putida* S12.

It was shown that *P. putida* S12 adapt to the presence of organic solvents by isomerization of *cis* to *trans* unsaturated fatty acids in the membrane-lipids². Recently, we demonstrated that *P. putida* S12 possesses an active efflux system exporting toluene out of the cell³. The nature of this active efflux system is still poorly understood. However, it may have features in common with the multidrug resistance studied for antibiotics⁴.

Therefore, we studied now if the tolerance of *P. putida* S12 to organic solvents is also connected with the antibiotic resistance.

The process of the solvent-adaptation of *P. putida* S12 was followed by cultivating the strain in the presence of increasing concentrations of toluene. We showed that the tolerance to various structurally and chemically not related antibiotics with different targets in the cell increased during this stepwise adaptation. After the adaptation to saturated concentrations of toluene the survival of *P. putida* S12 in the presence of antibiotics like tetracycline, chloramphenicol and piperacillin increased more than a factor 1,000.

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In the framework of the EC project "Terpene Biodegradation" we have been working on the strain M1 of *Pseudomonas* sp. which can grow on myrcene as sole carbon and energy source. The main purpose of our work is the production of myrcene oxidation products with important characteristics as fragrance and flavouring agents or for the production of fine chemicals.

The chosen strategy consists in the cloning of gene(s) coding for single enzymatic activities in a *Pseudomonas* strain unable to utilize myrcene. In this way single products will be obtained in each biotransformation process. Therefore, our work was directed in two main directions: construction of the M1 genomic library and isolation of M1 mutants unable to grow on myrcene. Moreover, being the M1 strain newly isolated, we have also investigated those characteristics that are important for carrying out the genetic work: phenotype stability, conjugation test, antibiogram and the presence of plasmids have been checked. The results obtained (data will be presented) showed that the M1 strain was suitable for further genetic work. Chemical and transposon mutagenesis have been carried out in order to obtain myrcene negative mutants. We have isolated two M1 mutants that have been partially characterized with the help of the biological oxygen monitor. The results indicate that at least one of them could be blocked in the first enzymatic step of myrcene conversion, since no oxygen consumption was observed in this mutant in the presence of myrcene.

Moreover, a genomic library of *Pseudomonas* sp. M1 has been constructed in a broad host range cosmid vector and amplified in *E. coli*. The *E. coli* clones were used for: a) direct identification of an oxygenase activity; b) complementation test with the previously isolated M1 mutants, and c) ability to confer the myrcene phenotype to a *Pseudomonas* strain unable to grow on this monoterpene. Up to now we have identified a chromosomal fragment of 9 kb which encodes an oxygenase activity.

The toxicity on the microbial growth of myrcene and of some of its oxidated-derivatives was tested in view of the use of recombinant *Pseudomonas* clones for the bioconversion of myrcene into flavour compounds.

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We have developed for the rapid and simple method for the detection and enumeration of *Pseudomonas putida* containing the mercury resistance genes (*mer* operon) using the polymerase chain reaction (PCR). Genetically engineered *Pseudomonas putida* PpY101/pSR134 was used in this experiment (1). Plasmid pSR134 was constructed by inserting two *EcoRI* DNA fragments encoding the *mer* operon from the NR1 plasmid into a broad-host-range vector pSUP104. The oligonucleotide primers were designed from a region of the *merA* gene that codes for the enzyme mercuric reductase. A direct PCR assay which amplified the target DNA from intact cells was performed. By using agarose gel electrophoresis for detection of the PCR-amplified products, the sensitivity of detection was 100 cells after 30 cycles of PCR and 1 cell after a 50-cycle double PCR. Quantitative PCR was applied using the ten-fold most probable number (MPN) scheme. The close correlation between total cell number and MPN-PCR data was observed. Then plasmid pSR134 was transformed into other *Pseudomonas* species. This direct PCR assay permitted the detection of amplified the target DNA from intact cells of pseudomonads.

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A large native plasmid is associated with the pathogenicity of *Pseudomonas syringae* pathovar *phaseolicola* towards bean

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We have obtained evidence for positively acting determinants of host range in the plant pathogen *Pseudomonas syringae* pv. *phaseolicola*. Eight of the nine races of *P. s. pv. phaseolicola* harbour a circa 150 kb plasmid, designated pAV505 in race 4 isolate 1302A, pAV530 in race 5 isolate 1375A and pAV511 in race 7 isolate 1449B. An origin of replication (*oriV*) of pAV505 has been previously cloned and characterised (1; M. J. Gibbon, A. Canal, A. Sesma, J. Murillo, and A. Vivian, unpublished results). The clone was electroporated into the nine races of *P. s. pv. phaseolicola* to attempt to evict native plasmids by incompatibility. Among the resulting electroporants of race 4 1302A, strains were identified that had been cured of the large native plasmid pAV505. A similar curing procedure used with the race 5 and race 7 strains resulted in curing of the native 150 kb plasmids pAV530 and pAV511, respectively. Loss of the plasmids did not correlate with any obvious change in colony morphology or culture appearance. Wild type and cured strains were inoculated into pods of compatible cultivars of the host plant *Phaseolus vulgaris*. Wild type strains caused disease typified by watersoaked lesions at the point of inoculation. However, strains cured of the 150 kb plasmid caused sunken brown lesions indicative of the plant defence (hypersensitive) response (HR). A race 7 gene library was constructed comprising 2000 clones and colony blots of these screened using pAV511 as a DNA probe. Forty eight strongly hybridising clones were identified and mobilised into the race 7 cured variants RW60 and RW63. Nine clones were observed to restore pathogenicity of RW60 and RW63 towards *P. vulgaris*.

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Pathway engineering for degradation of chloronaphthalene and other haloaromatics by *Pseudomonas* and *Burkholderia*

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The mineralization and detoxification of recalcitrant haloaromatic pollutants of the ecosphere is one of the major goals in environmental biotechnology. We constructed a *Pseudomonas putida* strain expressing the gene sequence *tcbR,C,D,E,F* from plasmid pP51, coding for a regulated chlorocatechol pathway which allows elimination of up to two chlorines from the haloaromatic ring system. In this case the manipulation enabled the organism to extend its capability for the mineralization of naphthalene to chloronaphthalene the degradation of which then occurred via chlorosalicylate. This new feature could not be achieved by horizontal gene transfer of chlorosalicylate degradative sequences and/or by mating techniques before since, by these methods, detrimental meta cleavage activity in the new host strains was not switched off. Moreover, the use of Tn5 minitransposons for chromosomal insertion allows the stable integration of gene sequences lacking an antibiotic resistance marker into the genome of strains of the genus *Burkholderia*, too; thus leading to the mineralization of mono- and dihalogenated derivatives of benzoate, phenol, salicylate, benzene, toluene, and biphenyl.

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We previously reported the isolation of *Pseudomonas* sp. JR1 using isopropylbenzene (IPB) as a new inducer substrate for cometabolic trichloroethene (TCE) degradation [1]. The genes encoding the first three enzymes of the IPB degradative pathway are *ipbA*/*A2A3A4* coding for the three subunits of a multicomponent IPB dioxygenase, which is responsible for the cometabolic oxidation of TCE [2], *ipbB* coding for dihydrodiol dehydrogenase and *ipbC* coding for 3-isopropylcatechol-2,3-dioxygenase.

For a successful application of the inducer dependent IPB and TCE degradation of strain JR1 an understanding of the IPB pathway regulation is invaluable. In order to address this question we generated deregulated mutants exhibiting constitutive catechol *meta*-cleavage activities. Interestingly, these mutants still exhibit inducible IPB dioxygenase activity.

Here we report on the cloning and molecular analysis of the wild type chromosomal locus of one such mutant, designated K1. Analysis of the nucleotide sequence of the contiguous DNA fragments flanking the kanamycin marker gene revealed two open reading frames, designated *armS* and *armR*. Sequence alignments of the deduced proteins revealed highest homologies to two-component systems belonging to the family of EnvZ/OmpR two-component regulatory systems. These results together with physiological studies of the mutant give evidence that the two-component system ArmSR regulates a catechol *meta*-cleavage dioxygenase in strain JR1.

Primer extension and RT-PCR experiments indicate that the constitutive catechol *meta*-cleavage dioxygenase is not encoded by the *ipbC* gene of the *ipb* operon. Further studies are underway to identify the *meta*-cleavage dioxygenase being regulated by the two-component system ArmSR.

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Two alkaliphilic bacteria were isolated from a highly alkaline site in Northern Ireland. Both organisms were found to utilise naphthalene as sole carbon and energy source at pH 9.5. A microbiological survey of the site showed that alkaliphiles were present in relatively high numbers (1.2×10^7 cfu/g at areas of pH 9.5-10.0), and some of these alkaliphiles could degrade naphthalene albeit slowly, suggesting that adaptation by exposure to PAHs may have occurred.

Biochemical testing indicated that both organisms were Pseudomonads, though not of the same species. One organism behaved as an obligate alkaliphile with no growth below pH 8.2, whilst the other was classified as a facultative alkaliphile, growing over the pH range 5.5 to 10.0. Currently, 16S rRNA sequencing is underway to further classify them.

Growth on naphthalene at pH 9.5 resulted in accumulation of metabolites which coloured the medium distinctively for each alkaliphile. The biomass of the alkaliphiles was found to be particularly low when growing on naphthalene compared to a mesophilic naphthalene degrading Pseudomonad (*P. putida* Pp G7). Presently, work is proceeding to establish the identity of the accumulating metabolites. Enzyme assays showed that both alkaliphiles appear to utilise naphthalene by the *meta* cleavage pathway for catechol. Catechol-2,3-oxygenase was induced by growth on salicylate, particularly in the facultative alkaliphile. Generally, the alkaliphiles had enzymes (including naphthalene dioxygenase) that exhibited a higher pH range than the mesophile. In addition, the facultative alkaliphile showed a wider pH spectrum for enzymatic activity than the obligate alkaliphile. This may be significant to understanding the alkaliphilic growth characteristics. Initial experiments indicate that both bacteria possess naphthalene dioxygenase genes with high homology to the gene in *Pp* G7.

A *Pseudomonas syringae* KCTC1832 outer membrane protein, ice nucleation protein (INP), has a unique structure which contains three domains : an N-terminal unique region (179aa), a central region (920aa) rich in alanine, glycine, serine and threonine residues bearing nested sets of repeated sequences, and a C-terminal unique region (49aa). We have developed a surface display system using this INP because it has a following characteristics as a display motif : 1) efficiently expressed on the outer membrane via the glycoposphatidylinositol(GPI) anchor, 2) stable at the stationary phase, 3) having the modifiable central repeating domain, 4) assembling into protease-resistant cluster, and 5) inter-Genus expression in Gram(-) bacteria. We constructed a surface display vector (pGINP21M) by replacing stop codon, TAA, with an oligonucleotide, 5'-GAT CCC GGG GAA TTC-3'(AspProGlyGluPhe), by PCR, in which 3 cloning sites (*Bam*HI, *Sma*I, and *Eco*RI) were newly created. This surface display vector could be used to construct an INP-foreign protein fusion if subcloned at one of those restriction sites.

For example, an open-reading frame of *Zymomonas mobilis* levansucrase (pSSTS110) was inserted at *Bam*HI and *Eco*RI sites. Transformed *E. coli* cells with pSSTS110 hydrolyzed efficiently sucrose and converted into a polyfructose(levan) and its monomer(glucose), suggesting that the INP-levansucrase hybrid protein was functionally expressed on the cell surface. Freely liberated glucose was used to support *E. coli* growth.

Therefore, this is more efficient system than the conventional method in which the enzyme is expressed in the cytoplasm and should be secreted into the growth medium. *Z. mobilis* sucrose gene was also fused to C-terminus of INP gene and expressed in *E. coli*. Growth of *E. coli* on sucrose by surface displayed sucrose activity was investigated.

The INP directed correctly foreign protein to the outer membrane surface if fused in-frame to the C-terminus of INP. This has been verified with several immunochemical experiments such as the confocal fluorescence microscopic observation, fluorescent-activating cell sorting (FACS) flow cytometry, and immunogold electron microscopic method. Several other proteins including carboxymethylcellulase (CMCase), chloramphenicol acetyltransferase (CAT), lipase, single chain antibody (scFv), etc. were also correctly displayed on the bacterial cell surface, thus demonstrating that the INP could be used as a novel surface display motif for foreign proteins.

Pseudomonas fluorescens SBW25 populations founded from single cells become highly polymorphic during stationary phase growth in structured environments, producing a diverse spectrum of niche-specific morphological variants. Previous studies documenting the ecological and evolutionary consequences of intracolon polymorphism in SBW25 populations have shown that phenotypic diversification displays characteristic dynamics; morphological successions exhibiting the features of both typological and temporal reproducibility. This study forms part of a broad initiative to account for adaptive radiation and ecological specialisation of SBW25 on the molecular level. Mutants unable to inhabit a defined ecological niche and produce the full wild-type repertoire of phenotypic variants were generated by transposon mutagenesis. Competition between mutant and wild-type populations in various environments demonstrated that the phenotypic target of selection has been identified.

Two loci essential for ecological specialisation and phenotypic diversification have been isolated and characterised. One locus spans 9kb and encodes genes with similarity to the cellulose synthase operon of *Acetobacter xylinum*. The second locus encodes a transcriptional activator similar to *pleD*, a transcriptional activator from *Caulobacter crescentus* involved in development. The nature of the genetic changes at these loci leading to adaptive evolution of SBW25 is the focus of current investigation.

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Majority of microorganisms in nature are not growing exponentially but are rather in a resting state or stationary phase of growth. We have designed a novel experimental system to study mutational processes in starving bacteria. This manifests in *de novo* creation of functional promoters that permit starving *Pseudomonas putida* cells to utilize phenol as a sole source of carbon. The promoters for transcription of the phenol degradation genes *pheBA* were created as a result of base substitutions, deletions and insertions of Tn4652 and novel IS element IS1411. All mutations observed by us created a sequence similar to σ^{70} -specific promoter consensus for the transcription of the *pheBA* genes.

Study of mutational processes in starving bacteria has revealed several unusual features. The spectrum of mutations that arise in a starving cell population under selective pressure is much narrower than that of mutations appearing during active growth of bacteria (1-3). These mutations seem to be promoted when the cells are starving in the presence of selective agent enabling escape from starvation. We found that the rate of accumulation of phenol-utilizing mutants on selective plates depends on the physiological state of the culture before plating, being higher in the case of using stationary phase cells. Presumably, certain DNA repair systems are impaired in starving cells. In our case, one particular C:G to A:T transversion was prevailing mutation type observed in *Phe*⁻ mutants that accumulated under starvation conditions whereas various deletions were the most frequent *Phe*⁻ mutants occurring in a culture growing without selection (4). The C to A transversion is known to be induced in *mutY* deficient cells (5). We propose that lower efficiency of MutMY repair pathway in starving cells would allow more DNA sequence alterations serving as material for fixation of useful mutation in these cells.

Many transposons tune their transposition frequency to the physiological state of the host (6). We found that Tn4652 transposition-generated *Phe*⁻ mutants appear only in starving cell population. The regulation of Tn4652-encoded transposase by repressor encoded by the same transposon will be discussed.

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Pseudomonas aeruginosa elastase, encoded by *lasB*, is synthesized as a preproenzyme with a 174 residue propeptide (Pro) which blocks elastase activity. Pro also functions as an intramolecular chaperone *in vivo*, required for elastase activity and secretion. To understand the mechanisms involved, we initiated *in vitro* studies of Pro-dependent elastase folding. Pro was produced in *P. aeruginosa* FRD740(Δ *lasB*) harboring a *lasB* allele with a stop codon at +1 of the mature sequence; it was then purified from the periplasmic fraction of these cells by adsorption to elastase-Sepharose. Purified Pro and mature elastase, denatured in guanidine-HCl, were combined, and renaturation assessed after dialysis and controlled trypsin treatment. Elastase activity was restored only in the presence of Pro, with maximal recovery of ~30% observed at Pro-elastase ratios \geq 1:1. Denatured elastase was degraded by trypsin. Immunoblots showed acquired resistance to trypsin of refolded elastase and dependence of elastase folding on interaction with Pro. Mutant unprocessed elastase precursors with deletions (ie., Δ -57 to +30, Δ -89 to +6, and Δ -164 to -12) were unfolded and accumulated in the membranes of expressing *E. coli* cells. When guanidine-HCl extracts of such membranes were mixed with Pro and tested for renaturation, only one (Δ -164 to -12), was folded into an active, trypsin resistant form. Apparently, certain propeptide sequences and/or deleted residues in the mature domain, interfered with elastase-Pro interaction in the other two mutants. Together, the present results show that Pro acts as a chaperone to promote folding of denatured elastase independently of other factors.

Organomercurial-resistance operon in a *Pseudomonas* K-62 plasmid pMR26.

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Pseudomonas strain K-62 was found to contain six plasmids. A mutant derivative cured of the 26-kb plasmid (pMR26) showed a higher sensitivity to mercurials. Two *SacI* fragments of the pMR26 that hybridized with the *mer* genes were cloned and expressed in *Escherichia coli*. One plasmid (pMRA17) inducibly encoded a typical broad-spectrum mercurial resistance, whereas the other plasmid (pMRB01) constitutively conferred hypersensitivity to phenylmercury (1).

DNA sequence of the broad-spectrum resistant *mer* operon was determined. The 5504-bp sequence of the pMRA17 includes six open reading frames (ORFs), five of which were identified as *merR*, *merT*, *merP*, *merA* and *merB1*, and the 2296-bp sequence of the pMRB01 includes three ORFs which were identified as *merR*, *merB2* and *merD* in order by analysis of deletion mutants and by comparison with the DNA and amino acid sequences of previously sequenced *mer* operons, respectively. The *merB1* encoding organomercurial lyase showed a less identity than the other *mer* genes with those from other broad-spectrum resistance operons, however the *merB2* showed a higher identity to the other known *merB*. The remaining ORF designated *merE*, located between *merA* and *merB1*, had no significant homology with the published *mer* genes and seemed to be a new gene which may be involved in phenylmercury resistance. Induction experiments and maxicell analyses of the *mer*-polypeptides revealed that pMRA17 *mer* operon expressed mercurial-inducible phenotype and the *merB1* and *merE* as well as the *merA* were under the control of *MerR*. The higher resistance to organomercurials shown by *Pseudomonas* strain K-62 may be achieved by the two functional *mer*-operons of pMR26 (2).

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95

Engineering of microorganisms for the degradation of recalcitrant chlorinated aromatics

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Chlorinated aromatic compounds represent a wide-spread class of environmental contaminants. Although microorganisms have evolved metabolic pathways capable of mineralizing some classes of chlorinated aromatics, there remain highly toxic environmental pollutants, such as the chlorinated dibenzo-p-dioxins, for which no microorganism capable of complete degradation has been isolated. To take dibenzo-p-dioxin as an example, the bacterium *Sphingomonas* sp. RW1 mineralizes unchlorinated dioxin but accumulates chlorinated catechols when grown on chlorinated dioxins due to the absence of a degradative pathway capable of handling chlorocatechols (1). We are attempting to expand the degradative potential of *Sphingomonas* sp. RW1 by equipping it with a functional chlorocatechol ortho cleavage pathway. The complementation of metabolic pathways through "laboratory evolution" should be a generally-applicable strategy for the expansion of biodegradative potential.

Cassettes of the chlorocatechol degradative genes (the *tcb* operon) from the trichlorobenzene-degrading bacterium *Pseudomonas* sp. P51 (2) have been created for monocopy insertion into bacterial chromosomes. The cassettes employ various promoters to drive expression of the *tcb* genes. Gene expression of chromosomally-inserted *tcb* genes has been tested from the natural inducible *tcb* promoter, as well as from fusions of the *tcb* structural genes with the inducible *sal* (salicylate) and *trc* (*trp-lac* hybrid) promoters. All three cassettes function effectively in *Pseudomonas* and *Alcaligenes* strains, and endow these strains with the ability to grow on chlorobenzoate or chlorosalicylate; however, expression of chlorocatechol-degrading enzymes has not yet been detected in *Sphingomonas* sp. RW1. In an attempt to obtain broad-host range expression of the chlorocatechol cassette, fusions of the *tcb* genes with a variety of constitutive promoters known to function in a range of Gram-negative bacterial species are being created and characterized.

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96

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Three multidrug efflux systems have been characterized so far in *Pseudomonas aeruginosa*. The MexE-MexF-OprN efflux system, which is overexpressed in *nfxC* type mutants, confers resistance to chloramphenicol, trimethoprim and quinolones. We have identified an ORF of 304 aa, located upstream of the *mexE-mexF-oprN* operon. Overexpression of this ORF from a plasmid was sufficient to confer a multidrug resistance phenotype on the wild type strain PAO1. A 34 kDa protein, which was called MexT, was expressed in *E. coli* from the ORF using a T7 polymerase system. The amino acid sequence of MexT was 30% identical to Nahr and NodD, involved respectively in activation of the *nah* catabolic operons in *Pseudomonas putida* and of the *nod* genes in *Rhizobium* sp. A *mexE::lacZ* fusion harboured on plasmid pMP220 yielded about 25 fold higher LacZ levels in *nfxC* type mutants than in the wild type strain PAO1. In *E. coli*, plasmid encoded MexT was able to transactivate the *mexE::lacZ* fusion by a factor of about 40. It is concluded that MexT is the transcriptional activator of the *mexE-mexF-oprN* efflux operon in *P. aeruginosa*.

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Polycyclic aromatic hydrocarbons, such as naphthalene, phenanthrene, anthracene, pyrene and others are a class of compounds regarded as ubiquitous pollutants. Thirty strains capable of utilizing naphthalene as a sole carbon and energy source have been isolated from the soil contaminated by oil products and wastes of chemical production. All strains were able to transform phenanthrene. However, only two strains appeared to be capable of utilizing phenanthrene as a sole carbon and energy source. But after prolonged cultivation on phenanthrene we also obtained five strains able to grow on this compound. For detail analysis we chose three most active phenanthrene-degrading strains: *Pseudomonas putida* BS3701, *Pseudomonas putida* BS3710 and *Pseudomonas* sp. BS3702. We have shown that *P. putida* strains BS3701 and BS3710 degrade phenanthrene to 1-hydroxy-2-naphthoic acid, which is further metabolize through salicylate and catechol. However, during growth of BS3701 on phenanthrene two intermediates accumulated. These compounds were identified as 2-hydroxy-1-naphthoic acid and dihydroxy-2-naphthoic acid by HPLC and mass-spectroscopy analysis. It was shown that degradation of phenanthrene and 1-hydroxy-2-naphthoic acid by BS3701 is determined by both plasmid and chromosome while degradation of these compounds by BS3710 is determined by genes located on NAH-like plasmid pBS216. In spite of the fact that BS3702 was unable to use exogenously supplied 1-hydroxy-2-naphthoic acid as a sole carbon and energy source, specific growth rate of this strain on phenanthrene higher than one for strain BS3701 which are capable to grow on 1-hydroxy-2-naphthoic acid. Knowledge of biochemical pathways and genetic control of polycyclic aromatic hydrocarbons biodegradation could provide further constructing and using effective xenobiotics degraders for bioremediation technologies.

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Many Gram-negative bacteria are able to translocate proteins across both membranes of the cell envelope into the extracellular medium. A number of these proteins are first transported via the Sec-machinery into the periplasm, where they obtain a considerable or complete tertiary conformation. In a second step the folded proteins are transported across the outer membrane. In *P. aeruginosa*, 12 *xcp* genes have been identified that are essential for this second step. Only one of the *Xcp* components, *XcpQ*, is located in the outer membrane and is therefore expected to form the protein translocation channel. *XcpQ* was purified and shown with electron microscopy to form a highly stable ring-shaped multimeric complex with an apparent central cavity. The observed cavity would be sufficient to allow transport of completely folded proteins. Homologues of *XcpQ* are involved in various processes, such as DNA uptake, type IV pili formation, contact secretion and the formation of filamentous phages. This fact implies that all these different processes depend on similar large transport channels. This notion was confirmed by showing that the PilQ protein, involved in type IV pili assembly, formed also a ring-shaped complex.

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Bacterial polyhydroxyalkanoates (PHAs) are polyesters of 3-hydroxyacids produced as intracellular granules by a large variety of bacteria. The best known examples are the short-chain-length (SCL) poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV) and copolymers of PHB/V. SCL-PHAs are brittle, rigid materials, analogous to polypropylene. Medium-chain-length (MCL) PHAs, which are mainly synthesized by *Pseudomonas* consist of monomers of 6 to 14 carbons, which are flexible polyesters. The PHA monomer composition is influenced by the substrate added to the growth media and determines the physical properties of the plastic. Inclusion of different functional groups into PHA can extend their physical properties and potential applications. PHA polymerase is the key enzyme of the polymerization reaction. *Pseudomonas oleovorans* GPo1 contains two different PHA polymerases encoded by *phaC1* and *phaC2*. Antibodies against polymerase C1 were raised and used to determine polymerase C1 levels *in vivo*. Furthermore, the incorporation rates of new monomers into preexisting PHA granules were quantified. When *P. oleovorans* GPo1 was grown under nitrogen limiting conditions growth stage dependent incorporation rates were found, with highest PHA synthesis rates of 9.5 nmol monomers / mg CDW * min. In addition to the *in vivo* experiments, an *in vitro* activity assay was established for PHA polymerases. CoenzymeA was enzymatically coupled to 3-hydroxyoctanoate for synthesizing the substrate 3-hydroxyoctanoyl-CoA. A direct correlation was found between 3-hydroxyoctanoyl-CoA substrate depletion and PHA synthesis due to polymerase C1 activity. Highest activity of 1.14 U/mg granule bound protein and highest specific activity of 2.8 U/mg polymerase C1 were determined. Polymerase C1 showed no activity towards 3-hydroxybutyryl-CoA and a specific activity of 0.28 U/mg polymerase C1 for hydroxyvaleryl-CoA.

Heterologous expression of the PHA synthase genes *phaC1* and *phaC2* from *Pseudomonas aeruginosa* confers PHA synthesis and accumulation to *Escherichia coli* LS1298

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In order to investigate recombinant PHA synthesis composed of 3-hydroxyfatty acids with medium chain length (C5-C14) and to analyse substrate specificity of *PhaC1* and *PhaC2*, we functionally expressed *phaC1* and *phaC2* in *Escherichia coli* LS1298. The *phaC1* and *phaC2* coding regions were amplified applying PCR and introducing the restriction sites *NdeI* and *BamHI* at the N terminus and the C terminus, respectively. The resulting PCR products were subcloned into the *NdeI* and *BamHI* sites of vector pT7-7. This construct caused the formation of inactive inclusion bodies in *E. coli* BL21 (DE3, pLys). To enable functional expression, the coding region of *phaC1* and *phaC2* were further subcloned by *XbaI* hydrolysis into the *XbaI* site of vector pBluescript SK⁺. The resulting plasmids pBHR71(*phaC1*) and pBHR71-C2(*phaC2*) caused functional expression of either PHA synthase under *lac* promoter control in *E. coli* LS1298 as monitored by PHA accumulation. This *E. coli* *fadB* mutant LS1298 has no detectable activities of enzymes involved in fatty acid β -oxidation. Fatty acids with chain length from C8-C14 were applied as carbon sources. *E. coli* LS1298 harboring plasmids either pBHR71 or pBHR71-C2 and cultivated in LB medium containing 0.5% (w/v) fatty acid revealed accumulation of PHA contributing to about 20% of the cellular dry weight. Analysis of PHA composition revealed that 3-hydroxydecanoate and 3-hydroxydodecanoate were the major constituents.

Adaptation of a *Pseudomonas* sp. isolated from food processing equipment to benzalkonium chloride

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Quaternary ammonium compounds (QACs) are commonly used disinfectants in food processing industry and hospitals because of their low toxicity, non-corrosiveness and high surface activity. Intrinsic and acquired resistance to these compounds, specially among Gram-negative bacteria, is well known.

Samples for isolation of bacteria resistant to QACs were taken from poultry processing equipment by swabbing. The samples were inoculated in a 1% solution of a commercial disinfectant containing benzalkonium chloride (BC). A *Pseudomonas* sp. which survived and multiplied in this solution was isolated. The isolated strain (Ps2) was further studied for acquisition and loss of acquired resistance to BC.

Ps2 was inoculated in tryptone soya broth (TSB) containing a sub-lethal concentration of BC (100 ppm) and bactericidal tests performed after 5 min, 30 min, 1 h, 2 h and 4 h of incubation respectively. The results showed that the adaptation process occurred during the lag phase. Bactericidal tests on Ps2 grown in TSB with 200 ppm BC over night and then reinoculated in TSB without disinfectant were performed. After 4 to 8 h of incubation without BC the strain lost the acquired resistance to BC. The resistance to the bactericidal effect of BC increased after incubation of Ps2 in the presence of relatively low concentrations of BC (less than 10 ppm). The mechanism of resistance to QACs is not fully understood. These results show that *Pseudomonas* sp. can adapt to QACs relatively fast and that the adaptation is most probably a regulated process.

The Role of Quorum Sensing in *Pseudomonas fluorescens* F113 DAPG Biosynthesis

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Pseudomonas fluorescens F113 produces an antifungal metabolite 2,4-diacetylphloroglucinol (DAPG) with important agricultural applications. This study is aimed towards a better understanding of the regulation of DAPG biosynthesis. Previous work has provided evidence that the expression of DAPG is cell density dependent and thus might be regulated through a "quorum sensing system". Quorum sensing systems characterised in other bacteria depend upon the accumulation of the autoinducer molecule N-acyl-L-homoserine lactones (AHL), the synthesis of which are directed by the regulatory proteins, the AHL synthase (a member of the *luxI* family) and the response regulator (in the *luxR* family). AHL molecules consist of a homoserine lactone moiety linked to a variable acyl sidechain. Variations in the length of the sidechain are produced by different bacteria and are associated with different biological properties. To begin this study our approach was to determine whether *P. fluorescens* F113 produces an AHL. To detect AHL activity, we routinely use several types of assays utilising biosensor strains which, producing either pigment or light, respond specifically to heterologous AHLs with either long or short acyl sidechains. The results of these assays revealed the presence of both a long and a short chain AHL.

Work continues to definitively identify these molecules. We are now cloning the genes of the *luxI/luxR* homologues by the strategy of complementation. Using these cloned genes we can construct a mutant defective in the *luxI* gene to establish whether a quorum sensing system is associated with the biosynthesis of DAPG.

103

Tn5501 and Tn5502, cryptic transposons of the Tn3 family resident on the plasmids of *Pseudomonas putida* strain H

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Genes encoding (methyl)phenol degradation of *Pseudomonas putida* strain H (*pht*) are located on plasmid pPGH1. Adjacent to the *pht* catabolic operon we identified a cryptic transposon, Tn5501, of the Tn3 family (class II transposons). Genes encoding the resolvase and the transposase are transcribed in the same direction as common for the Tn501 subfamily. Enzymes encoded by Tn5501, however, show only the overall homology characteristic for resolvases and transposases of Tn3-type transposons. Therefore, it is supposed that Tn5501 is not a member of one of the defined subfamilies.

Inactivation of the conditional lethal *sacB* gene was applied to detect transposition of Tn5501. During screening for transposition events we found another transposon integrated into *sacB* in one of the sucrose resistant survivors. This element, Tn5502, is a composite transposon consisting of Tn5501 and an additional piece of DNA. It is flanked by inverted repeats identical to that of Tn5501 and the additional part is separated from Tn5501 by an internal repeat (identical to the left terminal repeat).

Southern hybridizations show that plasmid pPGH1 carries one copy of both Tn5501 and Tn5502. On plasmid pPGH2 two copies of Tn5501 are located in a DNA region duplicated in an inverted manner. The arrangement of Tn5501 on pPGH2 might indicate that the transposon is involved in rearrangements of the genetical material. Transposition of phenol degradation genes could not be detected. Analysis of sequence data revealed that *pht* genes are not located on a Tn5501-like transposon.

104

Disulphide bond formation in *Pseudomonas aeruginosa*: Cloning and functional analysis of *dsbA*

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Many secreted proteins contain disulphide bonds which stabilize their active conformation. In *E. coli* disulphide bond formation takes place in the periplasm and is catalysed by at least five different Dsb-proteins [1,2]. *P. aeruginosa* is known to produce a number of extracellular enzymes, however, Dsb proteins have not yet been described. Based on a sequence comparison of known DsbA proteins we have designed degenerated primers which were used to amplify by PCR a 376 bp fragment of chromosomal *P. aeruginosa* DNA. This fragment was used as a homologous probe to identify a 2.3 kb *Xho*I fragment containing the *P. aeruginosa dsbA* gene which encodes a protein of 210 amino acids (M_r:23,300) and exhibits 68 and 21 % homology to DsbA proteins from *Azotobacter vinelandii* and *E. coli*, respectively. A truncated DsbA protein lacking the N-terminal signal sequence was overexpressed in *E. coli* as a His-tagged fusion protein and subsequently purified to electrophoretic homogeneity by immobilized metal affinity chromatography (IMAC). At present, we investigate the influence of purified DsbA on *in vitro* refolding of denatured *P. aeruginosa* lipase.

A *P. aeruginosa* mutant was constructed carrying an insertion of an Ω -fragment in the *dsbA* gene. This mutant showed a drastically reduced motility which could be restored by complementation with a plasmid harbouring the *dsbA* gene. Enzyme activities of alkaline phosphatase, elastase, and lipase were significantly reduced in the *dsbA* mutant suggesting that a functional DsbA is required for folding and/or secretion of enzymatically active proteins in *P. aeruginosa*. The influence of *dsbA* overexpression on the yield of extracellular lipase and elastase is currently studied. Furthermore, we try to identify in *P. aeruginosa* a *dsbC* gene presumably encoding a disulfide isomerase.

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105

Evidence of Physical Interaction between the PvdS Protein of *Pseudomonas aeruginosa* and the Core RNA Polymerase

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In *Pseudomonas aeruginosa* the biosynthesis of the siderophore pyoverdinin is strictly iron-regulated. The transcriptional control of the pyoverdinin biosynthetic genes *pvdA*, *pvdD* and *pvdE* is indirectly mediated by the iron-activated Fur protein through the repression of the *pvdS* gene (1, 2). The PvdS protein shows similarity with σ^{F} -like transcription factors, suggesting that it acts as an alternative σ factor for pyoverdinin genes (1, 3). Experiments were performed aimed to purify PvdS and to establish whether this protein behaves like a σ factor *sensu stricto*. Two different affinity chromatography systems were used. PvdS was tagged at the C-terminus with a 6xHis tail (originating PvdS6H), and with the FLAG octapeptide (originating PvdSF). Expression in *Escherichia coli* of the tagged PvdS proteins causes transactivation of the otherwise silent *pvdA*, *pvdD*, and *pvdE* promoters at the same level as the wild-type PvdS, demonstrating that both PvdS6H and PvdSF proteins retain functional activity. The tagged proteins were overexpressed in *E. coli* and recovered mainly as insoluble cytoplasmic fraction. PvdS6H was purified from inclusion bodies under denaturing conditions using a Ni-NTA resin, while PvdSF was purified under non denaturing conditions from the soluble fraction using anti-FLAG affinity gel chromatography. Under the non denaturing conditions, PvdSF was co-purified with the core fraction of the RNA polymerase (RNAP), as determined by Western-blot analysis with antibodies anti-FLAG and anti- α subunit of RNAP. Moreover, the PvdSF:core ($\sigma^{70}\beta\beta'$) RNAP complex copurified in a 1:1 stoichiometry. *In vitro* DNA-protein interaction experiments were carried on with the PvdS-RNAP complex. Gel retardation assays carried on with both PvdS6H and PvdSF showed that purified PvdS confers to core RNAP binding specificity to the *pvdA* promoter. These results provide strong evidence that the transcriptional activator PvdS behaves as a sigma factor.

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106

Rheological interactions between lipopolysaccharides of *Pseudomonas syringae* pathovar *coriandricola* and plant polysaccharides

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Phytopathogenic pseudomonads are mostly characterised by a narrow host specificity. For example *Pseudomonas syringae* pv. *coriandricola* (Psc) causes only diseases on *Coriandrum sativum* and *Levisticum sativum*.

Psc and many other pathogens of *Pseudomonas syringae* cause water soaked leave spots on their host plant. *Coriander* leaves show this symptoms only on young leaves in the rosette stage. The bacteria excrete exopolysaccharides (EPS) into the intercellular space of the plant where a highly viscous or gel-like matrix surrounds the bacterial cells. This gel allows continuous bacterial multiplication.

The EPS contain alginate, levan and lipopolysaccharides (LPS). The LPS may be one of the factors responsible for the highly specific host-pathogen-interaction.

In our studies we examined rheological interactions between the LPS of Psc and polysaccharides extracted from the host plant. We tested young and old leaves of *Coriandrum sativum*. Additionally polysaccharides from young leaves of *Petroselinum crispum*, a non-host-plant, were included.

LPS and pectins were examined separately and in mixtures of different proportions. The rheological investigations were carried out by a Brookfield DV III rheometer. We measured the shear stress of the solutions depending on different shear rates. With these data the viscosity and the yield stress of the mixtures were calculated.

The data of the interactions between LPS and polysaccharides of the host-plant revealed that the molecules interacted in a way that increased viscosity and yield stress. Mixtures of the two components were more viscous and more gelly than in the separate condition, indicating a synergistic interaction. Investigations on the interaction between LPS and polysaccharides of non-host-plants showed an opposite effect. In these mixture the viscosity and yield stress decreased, characteristic for an exclusion effect.

Role of four genes from *Pseudomonas fluorescens* in the biosynthesis of pyrrolnitrin

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Pyrrolnitrin (Pm) [3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole] is a secondary metabolite with strong antifungal activity that is produced by many *Pseudomonas* and *Burkholderia* sp. strains. Production of Pm is an important factor in the ability of some *Pseudomonas* strains to control plant diseases caused by soil-borne fungal pathogens. In addition, Pm has been used as a clinical antifungal agent for the treatment of skin mycoses and a phenylpyrrole derivative of Pm has been developed as an agricultural fungicide. We have cloned and characterized from *P. fluorescens* strain BL915 four genes, *pmABCD*, that together encode the biosynthesis of Pm (1). Based on the results of radiotracer studies and isolated metabolites, several possible biosynthetic pathways for the synthesis of Pm have been proposed. All proposed pathways agree that L-tryptophan is the precursor for Pm synthesis. We have constructed gene deletion/disruption mutations in each of the four *pm* genes and we have fused the coding sequence of each of the genes to promoter elements that are active in *Pseudomonas* in order to be able to study the functions of each of the proteins encoded by these genes. Analysis of metabolites accumulating in each of the four Pm-nonproducing mutants provided initial indications of the role of each of the genes in Pm synthesis. Cross-feeding of the deletion/disruption mutants with specific intermediates of Pm synthesis and of a strain lacking all chromosomal *pm* genes, but expressing individual plasmid-borne *pm* genes, confirmed the functions of the proteins encoded by the four *pm* genes. The *pmA* gene encodes a tryptophan halogenase that catalyzes the first step in Pm synthesis, the chlorination of tryptophan to form 7-chlorotryptophan (CT). The protein encoded by *pmB* catalyzes the ring rearrangement and decarboxylation of CT to form monodechloroaminopyrrolnitrin (MDA). The protein product of the *pmC* gene is a halogenating enzyme that catalyzes the chlorination of MDA in the 3 position to produce aminopyrrolnitrin (AP). The last step in Pm synthesis, the oxidation of the amino group of AP to a nitro group to form Pm, is catalyzed by the protein product of the *pmD* gene. These results confirm the pathway for the biosynthesis of Pm as proposed by van Pée et al. (2).

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The detection of insertion sequences within *Burkholderia mallei* and *Burkholderia pseudomallei* which have been identified previously in *Burkholderia cepacia*.

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Using five primer sets designed previously for the study of insertion sequences (IS) in *Burkholderia cepacia*¹, the presence of two IS were detected in the human pathogens *Burkholderia mallei* and *Burkholderia pseudomallei*. These IS correspond to IS406 and IS407 from *B. cepacia*. The IS407 homologue was cloned from both *B. mallei* and *B. pseudomallei* and sequenced to confirm its identity and degree of identity with IS407 from *B. cepacia*. A PCR amplification product using the *B. pseudomallei* strain NCTC 4845 as a template and IS407 primers, generated an IS407 probe which was used to determine the number and location of copies of IS407 in nine strains of *B. pseudomallei* by Southern Blot as compared to the numbers and locations of copies found in representatives of the four genomovars of *B. cepacia*. The nine strains of *B. pseudomallei* tested contained a mixture of clinical and environmental isolates including two environmental strains which differ from "standard" *B. pseudomallei* by three base changes in their 16S sequence.

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Regulation of Expression of the *bkd* operon of *Pseudomonas putida*

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Branched chain keto acid dehydrogenase (BCKAD) is the second enzyme in the catabolic pathway of branched chain amino acids (BCAA) and it is encoded by the *bkd* operon in pseudomonads. BCKAD is induced by the addition of BCAA or branched-chain keto acids to the growth medium. Expression of the *bkd* operon is positively regulated by BkdR, which is encoded by a structural gene which is divergently transcribed from the *bkd* operon. BkdR is a homolog of Lrp, the leucine-responsive protein of *Escherichia coli* with 36.5% amino acid identity to Lrp. Lrp is a global regulator in *E. coli*, but the low copy number of BkdR suggests that its main function is the regulation of the *bkd* operon in *P. putida*. Millimolar concentration of L-BCAA cause a conformational change in BkdR, and the fact that L-BCAA also affect the Dnase I protection pattern suggested that L-BCAA are the inducers of the pathway rather than branched-chain keto acids. Molecular mechanisms controlling the transcription of the *bkd* operon were elucidated using hydroxyradical footprinting and *in vitro* transcription techniques, and reported in this study.

The overall boundaries of BkdR binding to substrate DNA determined by hydroxyradical footprinting and DNaseI protection assay methods were the same, which revealed that BkdR is bound to DNA even after conversion to the active conformation upon L-BCAA binding. The periodicity of DNA binding pattern by BkdR evidenced from hydroxyradical footprinting suggested that the protein was bound to once face of the helix, probably along the major groove. *In vitro* transcription from the *bkd* promoter was demonstrated with sigma-70 saturated *E. coli* RNA polymerase (RNAP), BCAA, supercoiled template DNA and BkdR. The half of BkdR concentration at which maximum transcription occurs was $0.095 \times 10^{-6} \text{M}$ which corresponds to the cellular concentration of BkdR. A significant increase in transcription was found up to 15 mM L-valine concentration. The effect of BkdR and L-valine concentrations on *in vitro* transcription followed Michaelis-Menten kinetics. Although α -keto acids and D-enantiomers of both valine and isoleucine did not induce transcription, L-BCAA and D-leucine were equally effective in inducing *in vitro* transcription from the *bkd* promoter. The intensity and position of hypersensitive sites of DNaseI protection pattern with D-leucine and L-BCAA were similar suggesting that both ligands bind to the same site of BkdR. The conformationally active form of BkdR upon BCAA binding probably acts like a double clamp, bringing several binding sites close together forming a large nucleoprotein complex with RNAP to favor open complex formation. Deletion of few nucleotides of BkdR binding sites in the 5'-end of *bkdR* coding region resulted in: (i) a significant reduction of the *bkd* promoter activity, and (ii) a drastic reduction of *in vitro* transcription from the *bkd* promoter. This may be due to reduction of affinity of BkdR to substrate DNA, thus disabling the distal DNA to be brought close to RNAP for transcription. Thus, cooperativity among BkdR binding sites together with DNA bending, and the effector induced conformational changes of the regulatory protein are important in the positive regulation of the *bkd* operon. Although the expression of *bkdR* is constitutive, addition of glucose suppressed *bkdR* expression. Catabolite repression of the *bkd* operon can occur either by preventing binding of BkdR and/or interference with the transcription of *bkdR*, and the study of these factors is in progress.

Cloning and sequencing of the regulatory genes involved in the expression of the styrene degradative pathway in *Pseudomonas fluorescens* ST.

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Styrene is an aromatic hydrocarbon largely used for the production of plastics, synthetic rubbers and resins. For its massive use styrene is often found as environmental pollutant, being released either in the wastewater or in the wastegas outlet of the factories and, because of its toxic properties, the investigation of styrene-utilizing microorganisms is of great interest.

Pseudomonas fluorescens ST degrades styrene via the side chain oxidation which leads to the formation of the corresponding epoxide and, subsequently, to phenylacetic aldehyde and phenylacetic acid. The genes responsible for these enzymatic activities have been previously cloned, characterized and sequenced^{1,2}. A region of approximately 3 kb located upstream of the catabolic genes was found to be necessary for the expression of the degradative pathway. This region was cloned and completely sequenced. Two putative regulatory genes were found and were labelled as *styS* and *styR*. Both the nucleotide and the aminoacidic sequence of the two genes was compared against the EMBL data library and some relevant homologies were found. In particular the two gene products, StyS and StyR share significative homology with many members of the two-component regulatory system. The best alignments were found with the NodV/NodW proteins (modulation process in *Bradyrhizobium japonicum*), FixJ/FixJ proteins (nitrogen fixation in *B. japonicum* and *Rhizobium meliloti*) and DctS/DctR proteins (C4-dicarboxylates transport in *R. leguminosarum* and *Rhodobacter capsulatus*). From these data a similar regulatory model is proposed for the expression of the styrene catabolic genes. According to this model StyS could be the sensor protein which, in response to styrene, phosphorylates the StyR regulatory protein, making the latter functionally active as positive regulator of the *sty* genes transcription.

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Microbial degradation of chloroaromatics. Use of the *meta*-cleavage pathway for the mineralization of chlorobenzene

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Pseudomonas putida GJ31 is able to simultaneously grow on chlorobenzene and toluene. Usually, chlorinated aromatics are transformed to chlorocatechols, which are further metabolized by enzymes of the modified *ortho*-cleavage pathway, in which a catechol 1,2-dioxygenase opens the aromatic ring. Methylated aromatics are mostly mineralized via *meta*-cleavage pathways, in which a catechol 2,3-dioxygenase causes ring opening of the catechol. Simultaneous metabolism of chloro- and methylcatechols often creates biochemical anarchy. *Meta* cleavage leads to substrate misrouting in the case of 4-chlorocatechol, and the formation of a suicide acylchloride in the case of 3-chlorocatechol. Formation of dead-end methylsuccinates might occur when the *ortho*-cleavage pathway is dealing with methylcatechols.

The pathway of degradation of aromatic compounds in *P. putida* GJ31 was investigated by examining the presence of *ortho*- and *meta*-cleavage enzymes in crude extracts of cells grown on different substrates. No activity was found of enzymes of the (modified) *ortho*-cleavage pathway in extracts of chlorobenzene-grown cells of *P. putida* GJ31, while enzymes of the *meta*-cleavage pathway were active. 3-Chlorocatechol accumulated in the supernatant when cells growing on chlorobenzene were inhibited by 3-fluorocatechol. Since it was the sole metabolite from chlorobenzene, initial dechlorination, for example by formation of an unstable *gem*-alcohol, can be ruled out as a step in the degradation pathway of chlorobenzene.

Partially purified catechol 2,3-dioxygenase of *P. putida* GJ31 converted 3-chlorocatechol to 2-hydroxymuconic acid, which could be further degraded by the *meta*-cleavage pathway. The degradation route of chlorobenzene in *P. putida* GJ31 is given in figure 1 (Mars et al., submitted for publication).

P. putida GJ31 thus contains a catechol 2,3-dioxygenase which is able to resist suicide inactivation by the acylchloride formed during 3-chlorocatechol conversion, enabling it to use the *meta*-cleavage pathway to grow both on toluene and chlorobenzene. Currently, research focusses on differences between the catechol 2,3-dioxygenases of *P. putida* PaW1 and *P. putida* GJ31, enabling the latter to prevent suicide inactivation by the acylchloride that is expected to be formed from 3-chlorocatechol.

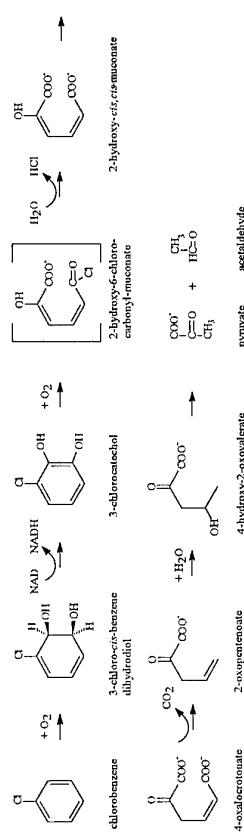


Figure 1: Novel pathway for the degradation of chlorobenzene in *P. putida* GJ31.

Characterization of Genes Essential for the Degradation of the Diterpene Dehydroabietic Acid by *Pseudomonas* BKME-9

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Dehydroabietic acid (DhA) is a tricyclic diterpene which is a component of wood resin. This diterpene is a waste byproduct of softwood pulping which causes acute toxicity of pulp and paper mill effluent and interferes with the paper making process. Tn5 mutational analysis of the biochemical pathway for the degradation of DhA by the *Pseudomonas* sp. BKME-9 produced a mutant which accumulated the metabolite 7-oxodehydroabietic acid (7-oxoDhA). An inverse PCR (IPCR) probe was produced from the mutant strain using Tn5 sequence as priming site. Nucleotide sequence analysis of the IPCR product indicated that the transposon had inserted into a region which had similarity to α subunits of several multicomponent dioxygenases. The DNA sequence of a 6-kb *EcoRI* fragment from a cosmid library clone had three complete open reading frames (ORF). Comparison of the deduced amino acid (a.a.) sequence of two ORF with translated sequences from GenBank database revealed homology to α and β iron-sulfur proteins of several dioxygenases. The two genes which are separated by a 54-bp non-coding segment were designated *DhaA1* (α) and *DhaA2* (β) and are 1407-bp and 201-bp, respectively. Deduced a.a. sequence comparison to other α and β subunits indicated that *DhaA1* has highest homology to *IphA1* (isopropylbenzene, 26%) and *BphA1* (biphenyl/PCB, 25%) genes whereas *DhaA2* showed highest homology to *BphE* and *BpdC2* (biphenyl/PCB, 37%). The highly conserved sequences for Rieske [2Fe-2S] cluster and mononuclear-iron binding sites are found in *DhaA1* subunit. Analysis of the sequence proximate to these genes did not indicate the presence of genes encoding a ferredoxin or a reductase but identified a potential stem-loop terminator, 39-bp downstream of *DhaA2*. The third ORF, designated *DhaB*, is located 913-bp (non-coding) downstream of *DhaA2*. Similarity analysis of *DhaB* revealed that this gene belongs to the family of short-chain dehydrogenase/reductase with the highest similarity to several hydroxy-steroid and dihydrodiol dehydrogenases. Disruption of *DhaB* in the wild-type strain by insertion of a Gm/XylE cassette indicated that this gene is required for rapid degradation of DhA and 7-oxoDhA and is inducible by both substrates. Resting cell suspensions of this mutant accumulated a hydroxylated metabolite in the presence of 7-oxoDhA but not DhA. Characterization of the enzymes encoded by *DhaA* and *DhaB* and their biochemical function is in progress.

113

Cloning and characterization of a functional homologue of the *P. aeruginosa* Type II protein export gene *xcpQ* and its role in exoprotein secretion

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Pseudomonas aeruginosa secretes a number of enzymes (lipase, phospholipase C, exotoxin A, elastase, and alkaline phosphatase) into the extracellular medium via a Type II secretion system. Once in the periplasm, transport of the enzymes across the outer membrane requires XcpP-Z and PilD/XcpA. Since there are many other proteins found in the periplasm that are not exported into the extracellular medium, there must exist a component(s) that recognizes the exoproteins and targets the protein to the rest of the export apparatus.

Studies have implicated homologues of XcpQ from other Type II systems as the "gatekeeper" of this export system. A mutant lacking *xcpQ* should be unable to export. However, we have shown that an *xcpQ* deletion mutant still exports at levels approximately 24 % of wild-type, suggesting that an additional gene product may be responsible for the residual level of export seen. A cosmid library was introduced into a *xcpQ* deletion strain and the resulting cells were screened for export levels above that of the parental strain. We have isolated a cosmid that restores export to the deletion mutant and have determined that it encodes a homologue of *xcpQ*. Disruption of this gene leads to loss of the residual export seen in the *xcpQ* deletion mutant.

Complementation by this gene restores export of lipase, alkaline phosphatase and phospholipase C, suggesting that if XcpQ is the "gatekeeper" its homologue is also capable of recognizing the same set of exoproteins. Interestingly, the coding sequence of the XcpQ homologue is unlinked to the other Type II export genes but is located adjacent to the gene encoding the staphylococcal LasA protease. The implications of these findings will be discussed.

114

Characterization of some physiological traits of *Pseudomonads*, potential PGPR or MHB, isolated from the mycorrhizosphere of *Suillus grevillei* (Klot.) Sing.

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It is known that *Pseudomonas* spp. can benefit plants through many different mechanisms of action. The re-establishment in the rizosphere of microorganisms possessing one or more beneficial characteristics is an important factor since it may influence plant growth and mycorrhization. Mycorrhizal infection may be enhanced by mycorrhization helper bacteria (MHB), commonly found in the mycorrhizosphere in different soils and plant-fungus associations (1). In previous studies, we have identified several *Pseudomonas* species from the mycorrhizae of *Suillus grevillei*-*Larix decidua* and analyzed their interactions with the mycobiont (2). In the present study, we have characterized some physiological activities of 8 *Pseudomonas* species to select strains as potential PGPR or MHB *in vivo*.

Antagonism against 9 species of phytopathogenic fungi, production of siderophores, cyanide, indol-acetic acid, and solubilization of phosphates were determined. Inhibition of phytopathogenic fungi on iron-rich medium was frequently less significant and strongly reduced as compared to that observed on low-iron medium. Addition of FeCl₃ to low-iron medium frequently reduced the extent of inhibition. Only one strain significantly inhibited phytopathogenic fungi on both media. The most frequent microscopic mycelial alterations observed in the phytopathogens were: granulation and vacuolization of the cytoplasm, intercalary and apical clamidospores production, apical regeneration. All the strains produced siderophores, indol-acetic acid and were able to solubilize phosphates. Only two species produced cyanide. Our results suggest that some *Pseudomonas* strain, able to stimulate the growth of the mycobiont and with peculiar traits, could be employed as PGPR or as MHB for the *S. grevillei*-*L. decidua* symbiosis.

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Oxygen radical induced phenotypic change from non-alginate producing to alginate-producing form of *Pseudomonas aeruginosa* in biofilms

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The leading cause of fatality in patients with cystic fibrosis (CF) is the chronic lung infection due to *Pseudomonas aeruginosa* growing as a biofilm in their lungs. Initial infection and colonization of the lungs appears to be due to the typical non-mucoid form. Upon infection the host immune system responds and attempts to clear the bacteria, and, consequently, *P. aeruginosa* becomes a constant target for phagocytes. Later mucoid variants appear *in vivo*, and become predominant, suggesting that alginate production may confer selective advantage to the bacteria. To date, no laboratory conditions have been identified that will convert non-alginate producing wild type *P. aeruginosa* into alginate-producing phenotype (Alg⁺).

Pseudomonas infection is often accompanied by a heavy infiltration of polymorphonuclear leukocytes. Upon activation due to infection, these cells generate large amounts of free oxygen radicals such as hydrogen peroxide, and release them into the local environment. In an attempt to mimic the environment of the neutrophil activated lungs, an Alg⁻ strain was cultured in a biofilm flowcell and repeatedly subjected to low concentrations of hydrogen peroxide. Several independent Alg⁺ strains were isolated from a prototypic Alg⁻ *P. aeruginosa* PAO1. The analysis of these mutants showed no detectable differences in lipopolysaccharide, antibiotic or DNA profiles compared to the parent strain. However, outer membrane protein analysis displayed a 54 kDa protein characteristic of alginate-producing *P. aeruginosa*. In the analysis for the production of homoserine lactone derivatives (Quoruminducer, molecules), increased levels of 3-oxo-dodecanoyl-homoserine lactone (OdDHL), a *lasI* product, were observed. However, no detectable amounts of butanoyl-homoserine lactone (BHL), a *rhlI* product, were observed. Further investigations of the mutants are underway.

It has been demonstrated that oxygen radicals can induce gene expression in plant and mammalian cells. These genes are proposed to protect the cells against the toxicity of the oxygen radicals. Our findings indicate that alginate production induced by oxygen radicals in *P. aeruginosa* may serve as a defense mechanism for the bacteria.

A gene cluster in the plant growth promoting *Pseudomonas fluorescens* WCS374 involved in the biosynthesis of salicylic acid and the new siderophore fluorebactin

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Pseudomonas fluorescens WCS374 produces, at low iron availabilities, the siderophore pseudobactin and salicylic acid (SA), which have been suggested to be involved in the suppression of Fusarium wilt in radish exerted by this bacterial strain (1). The isolation of pseudobactin-deficient Tn5 insertion mutants led to the observation that a second siderophore (fluorebactin) is produced by this strain. Several lines of evidence suggest that fluorebactin production and SA production are related, and that SA is a precursor in the biosynthesis of fluorebactin. We have been able to clone a region of the WCS374 genome (cosmid clone pMB374-07, 28 Kb) that contains the loci necessary for both SA and fluorebactin biosyntheses. Deletion and subcloning analysis showed that an internal 5 Kb *EcoRI* restriction fragment is solely responsible for SA biosynthesis. This fragment has been sequenced. The SA biosynthesis region contains four genes that have been named *fsbCEAB* genes (from fluorebactin biosynthesis). The predicted amino acid sequences of these genes show relevant homologies to: isochorismate synthases and other chorismate-utilizing enzymes (*FbsC*); siderophore biosynthesis genes such as enterobactin, yersiniabactin, and pyochelin (*FbsE*); histidine decarboxylases of different microorganisms which activity yields the biogenic amine histamine (*FbsA*) and, finally, to the PchB protein of *P. aeruginosa* involved in the biosynthesis of SA and the siderophore pyochelin (*FbsB*).

Preliminary mass spectrometry data obtained from fluorebactin reveal that both SA and histamine moieties are present in the molecule but the structure appears to differ from SA- and/or histamine-based siderophores already described (i.e. pyochelin, pseudomonine, anguibactin).

Production of fluorebactin has been detected in *P. putida* pseudobactin deficient mutants, upon transfer of the cosmid clone pMB374-07. Likewise, SA production has been achieved in *P. putida* and *E. coli* strains with different pMB374-07 derivatives. Reverse transcriptase-PCR (RT-PCR) has been used to study expression of the *fsb* genes, demonstrating that they are arranged in a polycistronic organization and that their expression is iron regulated. The role of SA, fluorebactin and pseudobactin in disease suppression by strain WCS374 is currently being investigated.

Reference:

1. Phytopathology 86 (1996) 149-155

The product of the open reading frame ORF G possibly interacts with XanB, a bifunctional enzyme with PMI/GMP activities in the sugar nucleotide biosynthesis of *Xanthomonas campestris* pv. *campestris*

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The Gram-negative bacteria *Xanthomonas campestris* pv. *campestris* produces the acidic xopolysaccharide xanthan that is used as a thickening agent in the food industry. The repeating unit of xanthan consists of the monosaccharides glucose, mannose and glucuronic acid and is substituted with succinyl- and acetyl-moieties in various extents. The precursors of xanthan are the sugar nucleotides UDP-glucose, UDP-glucuronic acid and GDP-mannose. Several genes of the nucleotide sugar- biosynthesis are clustered on a 35,5 kb *EcoRI* fragment on the *Xanthomonas* genome (1). Within this cluster the genes *xanA* and *xanB* are coding for phosphomannomutase (PMN) and phosphomannose isomerase/GDP-mannose pyrophosphorylase (PMI/GMP), respectively. They are involved in the formation of UDP-glucose and GDP-mannose (2). XanB is a bifunctional enzyme with PMI and GMP activity and is a homologue of AlgA of *Pseudomonas aeruginosa*. Inactivation of *xanA* and *xanB* leads to a nonmucoid phenotype due to the loss of xanthan biosynthesis. The analysis of other nonmucoid mutants located on this fragment revealed the open reading frame ORF G. Inactivation of ORF G leads to a drastic reduction of PMI/GMP (*xanB*) activities but only to a decrease in xanthan biosynthesis of about 40-50% after 10 days of growth in modified minimal media with 1% glucose as the carbon source. The analysis of transcriptional and translational fusions of the chloramphenicolacetyl transferase (CAT) with the gene *xanB* in the *Xanthomonas* wildtype strain B100 and the ORF G mutant H2321 showed no influence of ORF G on these regulatory levels. In this work we present genetic and phenotypic data of ORF G mutants that propose a direct interaction of the ORF G product and XanB. We suggest a model for the mode of action of the ORF G product in sugar nucleotide biosynthesis.

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Testosterone inducible expression of enzymes involved in steroid and aromatic hydrocarbon catabolism in *Comamonas testosteroni*

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The Gram-negative bacterium *Comamonas testosteroni* (formerly *Pseudomonas testosteroni*) is able to grow at the expense of steroids, bile acids and aromatic hydrocarbons (AHs) as unique source of carbon and energy. The catabolic enzymes of the degradation pathways are not constitutively expressed but induced by their respective steroid or AH substrates. Although metabolic pathways for steroids and AHs have been intensively studied, the question remains whether both classes are substrates of different degradation routes or whether some catabolic enzymes function in both pathways. The steroid molecule resembles an ancient molecule that occurs in multiple forms in the environment. It may therefore be hypothesized that steroids represent the original substrates of many of these enzymes. Moreover, steroids may be involved in the regulation of these inducible proteins. In our studies we used two dimensional gel electrophoresis to identify proteins regulated in their expression by the steroid testosterone in *C. testosteroni*. Comparing the resulting protein pattern of induced vs. control extracts, we identified at least eleven testosterone regulated proteins, showing a significantly increased expression after steroid treatment. Conversely, an amino acid degrading enzyme was repressed upon steroid presence. After N-terminal amino acid sequencing of the induced protein spots, database searches revealed strong similarities to enzymes involved in both, steroid and AH-degrading pathways; such as steroid dehydrogenase, aldehyde reductase, biphenyldiol dioxygenase/dihydroxy-naphthalene dioxygenase, hydroxyoxovalerate dehydrogenase, ketoadipate succinyl-CoA transferase, hydroxypentadienolate hydratase, hydroxyphenyl pyruvate dioxygenase. We conclude from our results that the expression of enzymes involved in both catabolic pathways of *C. testosteroni* may be subject to a common regulation, in which steroids may play a central role. Further experiments are in progress to elucidate steroid inducible operons in *C. testosteroni*.

Analysis of the diversity of high-affinity iron uptake systems in *Pseudomonas fluorescens* ATCC17400

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Pseudomonas fluorescens ATCC 17400 is able to utilise different exogenous siderophores (pseudobactine BN7, pseudobactine B10, ferrichrome, deferrioxamine E). A PCR amplification was done on *P. fluorescens* DNA using degenerate primers corresponding to conserved regions from different pyoverdine/pseudobactine receptors sequences (1). After amplification and cloning, 4 different clones were obtained. The sequences showed similarities with different known receptors for siderophores from fluorescent pseudomonads. A Tn5 mutant was obtained which did not produce pyoverdine but still produced two siderophores which are present at different times during the growth. One compound, identified as a chinaldic acid (2), and named quinobactine, was taken up much more efficiently by the mutant than by the wild type. A second siderophore was identified by TLC, which was produced by the mutant only after 48 hours of growth. The mutant also produces one or two supplementary iron-repressed high-molecular weight proteins not detected in the wild type. All these results point to great variety in the high-affinity siderophore-mediated iron uptake systems in *Pseudomonas fluorescens* ATCC 17400.

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Bicistronic *aacC1-lacZ* reporter transcriptionally fused to the catabolic *phl* promoter suitable to identify mutants defective in carbon catabolite repression of phenol degradation

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(Methyl)phenol degradation mediated by phenol hydroxylase and *meta* cleavage pathway enzymes encoded by plasmid pPGH1 of *Pseudomonas putida* strain H is subject to carbon catabolite repression (CR). The main mechanism is an inhibition (or inactivation) of the regulatory protein PhlR. Therefore, activation of the catabolic operon in response to the availability of the substrate does not take place as long as cells grow exponentially on the preferred carbon source (Müller *et al.* 1996). When cells reach stationary phase, however, the catabolic operon is slowly transcribed. To identify genes whose products are involved in CR of phenol degradation we built up the following reporter system. The bicistronic reporter cassette consisting of the promoterless genes *aacC1* (encoding gentamycin-(3)- acetyltransferase type I) and *lacZ* was transcriptionally fused to the catabolic *phl* promoter and together with *phlR* (transcribed under its native promoter) cloned in a mini Tn5 and inserted in single copy situation into the chromosome of a *Pseudomonas putida* strain devoid of any *phl* genes. Phenotype of cells carrying this reporter system differs depending on the growth substrate. Although on all carbon sources colonies turned blue with X-gal, they are only resistant to gentamycin when they grow with pyruvate (which does not provoke CR) as carbon source. When cells grow with glucose, TCA cycle intermediates and lactate as carbon source or on nutrient medium they are gentamycin sensitive. Selection of gentamycin resistant colonies therefore should be suitable to identify mutants in which CR is reduced or absent at all. On minimal medium containing any of the preferred carbon sources gentamycin resistant clones arise spontaneously. Some of them are due to up- mutations of *phlR* gene promoter. Gentamycin resistant colonies were also found following mutagenesis with mini-Tn5. Analysis of these mutants indicate that they express a different pattern of resistance on the various carbon sources.

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- Müller *et al.* (1996) *J. Bacteriol.* 178: 2030-2036

Identification, Cloning and Characterization of a *crc*-like Gene of *Pseudomonas putida*

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The *crc* (catabolite repression control) gene, firstly identified and described in *Pseudomonas aeruginosa*, is most likely involved in the regulation of various catabolic pathways (1). Mutations in the *crc* gene affect the succinate mediated carbon catabolite repression of e.g. amidase, mannitol transport and glucose-6-phosphate dehydrogenase. In order to identify a *crc*-like gene and to study the effects of a *crc*-mutation on carbon catabolite repression in *Pseudomonas putida*, PCR was performed using oligonucleotides deduced from the coding region of the *Pseudomonas aeruginosa crc* gene. Using genomic DNA of two different *Pseudomonas putida* strains as template fragments were amplified which had the same size as the fragment amplified from the *crc* gene of *Pseudomonas aeruginosa*. Southern hybridizations with one of these fragments as probe allowed the identification and cloning of a genomic fragment of a *Pseudomonas putida* KT2440 derivative. DNA sequencing of the cloned fragment and computer assisted analysis revealed the presence of an open reading frame of 777 bp highly homologous to the *crc* gene. The homology is about 82% on DNA level and about 86% on amino acid level. We therefore suppose that the open reading frame codes for a protein similar to the *Pseudomonas aeruginosa crc* gene product.

Reference:

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Properties of polyester polyurethane degrading enzyme from *Comamonas acidovorans* TB-35

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Polyurethane (PUR) is one of the plastics which are widely used as raw materials for various industries, but they are known to resist biodegradation. Previously, we reported the isolation of a bacterium, *Comamonas* (formerly *Pseudomonas*) *acidovorans* strain TB-35, which degrades polyester PUR as a sole carbon and nitrogen source. We also identified PUR breakdown products and found that PUR degradation by strain TB-35 was started by hydrolysis of ester bonds of polyester segments of PUR. Strain TB-35 produced two different extracellular esterases, one which is secreted to the culture broth and one which is bound to the cell surface. Between them, only cell-bound esterase catalyzes the degradation of the polyester PUR. The cell-bound PUR degrading enzyme, PUR esterase, was purified until it showed a single band in SDS-PAGE. Optimum pH of this enzyme was 6.5, and the optimum temperature was 45°C. PUR degradation of the PUR esterase was strongly inhibited by the addition of 0.04 % of deoxy-BIGCHAP. On the contrary, when *p*-nitrophenyl acetate, which was a water-soluble compound, was used as a substrate, the effects of deoxy-BIGCHAP were not observed.

These observations indicate that this enzyme degrades PUR by two steps of reaction, consisting of hydrophobic adsorption to the PUR surface and hydrolysis of ester bonds of PUR. The other esterase, which is secreted to the culture broth, of strain TB-35 was also purified. This esterase could degrade poly(diethylene glycol adipate), which consists of soft segments of the PUR, but could not degrade PUR. The physicochemical properties of this esterase resembled those of the PUR esterase. In addition, the N-terminal amino acid sequences (20 a.a.) of these esterases were identical. On the contrary, the hydrophobicity of this esterase was much lower than that of the PUR esterase. From these results, we consider that these two esterases consist of the same polypeptides but their three-dimensional structures are different.

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Isolation and characterisation of rhizosphere induced genes from *Pseudomonas fluorescens* using *in vivo* expression technology (IVET), incorporating GFP and *inaZ* reporters

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Pseudomonas fluorescens is a vigorous coloniser of the rhizosphere. Certain isolates of *P. fluorescens* are known to promote plant growth and therefore have potential as biocontrol agents. Unfortunately this potential has not been realised. One reason for this is a lack of understanding regarding the interactions that occur in the rhizosphere. In order to improve our understanding of the effect of the rhizosphere environment on gene expression in rhizosphere-colonising *P. fluorescens* we have developed two IVET (*in vivo* expression technology) strategies. These strategies enable the isolation of *P. fluorescens* genes responsive to rhizosphere signals. Our IVET systems are based on *panB* and *dapB*, and the knowledge that both pantothenate and diaminopimelic acid are severely limiting in the rhizosphere. *P. fluorescens* genes showing elevated levels of expression in the rhizosphere are isolated through their ability to drive the expression of either a promoterless *panB* or *dapB* gene. In both cases promoterless *lacZ*Y was used as a reporter for gene activity. We have modified these constructs to improve detection of gene activity both *in vivo* (on the plant) and *in vitro*. Promoterless copies of the green fluorescent protein from *Aequorea victoria* and the *inaZ* gene from *Pseudomonas syringae* have been substituted for promoterless *lacZ*Y and provide convenient and powerful reporters for gene activity both *in vivo* and *in vitro*. The modified constructs have been used to obtain *Pseudomonas* genes induced in the plant rhizosphere and on plant leaves.

Deletion and Self-mobilization of the Chromosomal Gene clusters Coding for Biphenyl and Salicylate Metabolism in *Pseudomonas putida* KF715

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Biphenyl-utilizing bacteria are ubiquitously distributed and considered to involve in the final degradation of the plant lignin together with other aromatic degraders. They possess similar biphenyl catabolic *bph* clusters, suggesting the transfer of *bph* genes in soil bacteria. The biphenyl and salicylate metabolic pathways of *Pseudomonas putida* KF715 are chromosomally encoded. The *bph* gene cluster coding for the conversion of biphenyl to benzoic acid and the *sal* gene cluster coding for salicylate *meta*-pathway were obtained from the KF715 cosmid genomic libraries. The *bph* genes of KF715 were similar to those of *P. pseudoalcaligenes* KF707. KF715 *bph* genes were deleted a 3.5 kb *bphX* region between *bphC* and *bphD* in KF707 *bph* genes. The *bph* and *sal* gene clusters were separated by ca. 10 kb DNA. The pathways were highly prone to deletion when KF715 was grown in rich medium. The deletion was taken place at the region (ca. 40 kb) including only *bph* genes (KF796), or the region (ca. 70 kb) including both *bph* and *sal* genes (KF791). The both *bph* and *sal* genes could be transferred by conjugation to *P. putida* AC30 at a frequency of 10^{-4} per donor cell. These gene cluster was integrated into the chromosome of AC30. Such transconjugants gained the ability to grow on biphenyl and salicylate as sole carbon sources. The immobilized region extended to ca. 90 kb which completely included the deletion regions in KF796 and KF791. Furthermore, the chromosomally integrated *bph* and *sal* genes in AC30 were also highly prone to deletion and were immobilized to the chromosome of *P. putida* KT2440. Insertion of transposon within the *bphD* region resulted in no deletion nor mobilization of the KF715 chromosome.

Soil bioremediation: Mineralization of pentachlorophenol upon inoculation with *Sphingomonas chlorophenolica* SR3 and abiotic amendment

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Augmentation of pentachlorophenol (PCP)-contaminated soil with the PCP-degrader, *Sphingomonas chlorophenolica* strain SR3 (1;2;3), was investigated in laboratory microcosms in view of possible applications in soil bioremediation. Soil from a PCP-contaminated site, possessing a high indigenous PCP-mineralization activity, was spiked with ^{14}C -labelled PCP at 30, 100, 300 and 600 mg/kg. Strain SR3 was inoculated into the spiked soil at 10^4 , 10^6 and 10^8 cfu/g. Mineral nutrients (phosphate and ammonium) and structural materials, including polyurethane foam (PU), bentonite, atapulgit, vermiculite, sepiolite or sawdust, were added along with the bacterial cells to enhance their survival or activity. The activity of the inoculum, defined as PCP-mineralization in excess of the indigenous activity, was determined by quantification of $^{14}\text{CO}_2$ evolved. Mass balances for mineralized plus residual PCP were performed by extraction and GC/ECD-analysis.

At 30 mg/kg PCP, the indigenous microbiota mineralized 65% of the initial PCP in approx. 14 weeks. Inoculation with strain SR3 reduced this timespan to approx. 3 weeks, depending on inoculation density. However, at 100–600 mg/kg PCP, indigenous mineralization was negligible, and inoculation with strain SR3 did not enhance its rate. While amendment with mineral nutrients had no effect on the activity of strain SR3, amendment with clay minerals and sawdust improved its activity at 30 mg/kg PCP. Immobilization of strain SR3 on PU prior to inoculation did not improve its effect at 30 mg/kg, but did at the higher PCP concentrations. Indeed, at 100–600 mg/kg PCP, the only treatments that showed technically usable mineralization rates were those with strain SR3 immobilized on PU. Possible mechanisms of the observed effect of PU will be discussed.

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Characterization and Regulation of an Operon for Transport of Arginine and Ornithine in *Pseudomonas aeruginosa*

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The arginine succinyltransferase pathway (AST) enables *P. aeruginosa* to utilize arginine and ornithine as the sole carbon and nitrogen source (1). The *aru* genes encoding enzymes of AST and a regulatory protein, ArgR, have been cloned and characterized (2-4). Upstream of argR we found five ORFs which encode proteins very homologous to the lysine-arginine-ornithine binding protein (LAO) and histidine transport permease proteins (HslJ, Q, M, P) of *Escherichia coli* and *Salmonella typhimurium*. The transport genes appear to comprise an operon together with argR; in the order of *aotQ*-*aotM*-*aotO*-*aotP*-*argR*. *AotJ* is homologous to the periplasmic binding proteins, LAO and HslJ (46 and 44% identity, respectively). *AotQ*, *AotM* and *AotP* have homology with HslQ, HslM and HslP, of 52, 48 and 70% identity, respectively. No protein similar to *AotO* was found in the data bases. Arginine induces uptake of arginine, ornithine and lysine in PAO. Lower levels of uptake of the amino acids were observed in $\Delta aotQ::aph$ mutant cells induced by arginine. The deletion derivative grew slowly compared to parent strain when arginine or ornithine was used as the sole carbon and nitrogen source. Studies with an *aotJ*::*LacZ* translational fusion indicated the presence of a promoter upstream of *aotJ*, the expression of which is induced by arginine and repressed by succinate. Quantitative S1 mapping revealed two promoters upstream of *aotJ* that are regulated differently by exogenous arginine. DNaseI footprinting showed the binding of ArgR to a region of 45 bp overlapping two promoter regions. The apparent dissociation constant for ArgR binding of *aot* operator was 7.2×10^{-11} M. Premethylation and depurination footprintings identified a sequence that is important for ArgR-*aot* interactions as is the case for the biosynthetic *car*, and *argF* operons (3) as well as the catabolic *aru* operon (3).

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Screening and characteristics of aromatic compounds utilizing microorganisms which can grow under low nitrogen and phosphorus condition

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For the waste water treatment of oil refinery, we screened microorganisms which can utilize aromatic compounds under low nitrogen and phosphorus conditions. Twenty two strains isolated from soil for the ability to utilize aromatic compounds under low nitrogen and phosphorus conditions. These microorganisms include some Gram-negative bacterial strains, one Gram-positive bacterial strain and one yeast strain. Aromatics utilizer, *Ralstonia pickettii* K11, degraded 100 mg/l of toluene, 80 mg/l of benzene and 50 mg/l of xylene for 5 days' cultivation. Gram-negative bacteria isolated as phenol utilizer, *Burkholderia cepacia* 1A and *Alcaligenes denitrificans* subsp. *denitrificans* K2, needed less than 1 mg/l of phosphorus and nitrogen for the cell growth and degraded 200 mg/l of phenol for 3 days' cultivation. In contrast, five American Type Culture Collection's strains of phenol utilizers required more than 500-1000 mg/l of phosphorus and nitrogen for the cell growth and degradation of phenol. It was suggested that our isolated strains belong to the oligotrophic bacteria, because some strains could grow in 1/1000 diluted nutrient broth. It was confirmed that strains 1A and K2 grew under low phosphorus and nitrogen conditions using phenol as sole carbon source, by microscopic method and variable cell count method. Further work is in progress for molecular analysis of the growth mechanism under low nitrogen and phosphorus condition.

A part of this work was conducted as industrial technology development promotion program of RITE for the global environmental problem.

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The inducible catabolic aliphatic amidase operon of *P. aeruginosa* consists of five genes *amiE*, *amiB*, *amiC*, *amiR*, and *amiS* regulated by a transcription antitermination mechanism. *AmiC*, the negative regulator of operon expression, is the 'amide sensor' of the regulatory circuit, and *AmiR*, the positive regulator, is an RNA binding protein. The crystal structure of *AmiC* has been determined and its overall fold is extremely similar to LivJ, the periplasmically located leucine/isoleucine/valine binding protein of *E. coli*. The amide binding site of *AmiC* lies within a cleft between the N and C terminal domains. Acetamide (inducer) binding causes the molecule to adopt a closed down structure (the 'on' configuration) whereas binding of butyramide (co-repressor), results in a more open structure (the 'off' configuration). *AmiR* the response regulator of operon expression functions to antiterminate a constitutively produced leader RNA transcript to allow full operon expression. *AmiR* shows no homology to other proteins in the databases and has proved to be very intractable to isolation/purification. It has now been possible to isolate a stable soluble *AmiC/AmiR* complex in the presence of butyramide. Using conventional purification techniques mg quantities of the complex have been isolated. The complex has been shown to disaggregate upon addition of acetamide and it thus appears that amide dependent regulation of *AmiR* activity occurs via a simple steric hindrance mechanism with *AmiC* occluding the *AmiR* RNA binding activity under non-inducing conditions.

We are currently investigating the mechanism of the transcription antitermination regulatory system by a study of the interactions within the *AmiC/AmiR* (protein/protein) complex and within the *AmiR*/leader RNA (protein/nucleic acid) complex.

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Pyoluteorin is one of three antifungal metabolites that contribute to the ecological interactions of *Pseudomonas fluorescens* Pf-5 in the rhizosphere. Nucleotide sequence analysis of a 24-kb genomic region required for pyoluteorin production has identified at least eight open reading frames (ORFs) that encode biosynthetic enzymes. The deduced gene products of the identified ORFs include at least two halogenases, a Type I polyketide synthase, a thioesterase, a dehydrogenase, and a peptide synthetase. The predicted amino acid sequences of the halogenases are similar to those of chlorinating enzymes in the pyrrolnitrin and the chlortetracycline biosynthetic pathways. Loci encoding halogenases participating in secondary metabolism have been identified only recently, and the catalytic mechanism of these uncharacterized enzymes is not known. Multiple sequence alignments of the halogenase loci within the pyoluteorin gene cluster has identified an N-terminal peptide motif possessing the secondary structure necessary for NAD cofactor binding, suggesting that halogenases are a subclass of oxidoreductase enzymes.

A presumed regulatory gene (*pluR*) also has been identified within the pyoluteorin gene cluster. The deduced *pluR* product exhibits similarity to several members of the LysR family of transcriptional regulators. A putative *PluR* binding site is present 5' to the first ORF of the pyoluteorin gene cluster and is likely to be an important promoter element for *PluR*-mediated transcriptional regulation. *PluR* is essential for pyoluteorin production and is not required for the biosynthesis of other antifungal metabolites produced by Pf-5. Therefore, *PluR* appears to be a specific activator of the linked pyoluteorin biosynthetic genes and contrasts with the two-component regulatory system GacA/ApdA (Corbell and Loper, 1995) and the stationary-phase sigma factor RpoS (Sarniguet *et al.*, 1995), which influence the production of all three antifungal metabolites produced by Pf-5.

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Function and regulation of expression of the imipenem-specific porin OprD of *Pseudomonas aeruginosa*

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The *Pseudomonas aeruginosa* outer membrane protein OprD is a porin facilitating specifically the uptake of basic amino acids, small peptides containing these amino acids and their structural analogue, the carbapenem antibiotic imipenem. Previously, a topology model was proposed. 16 β -strands were predicted, connected by short loops at the periplasmic side and eight external loops of variable length. OprD was the first specific porin that could be aligned with members of the non-specific porin superfamily. Deletion mutagenesis suggested that the external loop L2 is involved in imipenem binding. Additional deletion mutants of L2 were constructed covering almost the entire loop (34 amino acids). All plasmid encoded mutant proteins were expressed like the wild-type protein in *E. coli* and *P. aeruginosa*. Reconstitution of imipenem susceptibility was determined in an *oprD* deficient strain. The L2 mutants only partially reconstituted imipenem susceptibility (MIC 4 μ g/ml), while expression of the wild-type protein fully reconstituted susceptibility (MIC 0.5 μ g/ml) in comparison to the vector control (MIC 16 μ g/ml). This confirms the topology model regarding loop L2 and strongly supports the previous suggestion that L2 is involved in imipenem binding. Uptake of carbapenem antibiotics by OprD was shown previously to be dependent on a positively charged substituent on position 2 of the carbapenem molecule. Consequently, we exchanged by site-specific mutagenesis all negatively charged amino acid residues in loop L2 against uncharged residues and investigated imipenem susceptibility.

The regulation of *oprD* expression appears complex and we are investigating the gene loci involved. Transposon Tn501 mutants were selected for resistance to imipenem and the quinolone norfloxacin. Five mutants were further characterized and their phenotype matched the previously described *nfxC* multiple-antibiotic-resistant phenotype. In all mutants resistance to gentamicin and carbentellin was reduced, while resistance to imipenem, chloramphenicol and quinolones was increased. Analysis of outer membrane profiles revealed that the amount of OprD was reduced. There was also an increase in the amount of an outer membrane protein of an apparent molecular weight of 50 kD. The protein could be distinguished from OprM, which is known to be involved in active antibiotic efflux. Mapping and cloning of the Tn501 insertion locus is currently in progress. Another gene locus consisting of three open reading frames was described before. One of the putative genes, *opdE*, was proposed previously to participate in regulation of *oprD*-expression. However, inactivation of *opdE* by insertion of a *xylE-Gm^R* cassette did not influence imipenem susceptibility. Thus it is unlikely that *OpdE* is involved in *oprD* regulation. Instead, recent sequence comparisons rather suggest that *OpdE* belongs to the major facilitator superfamily of efflux proteins.

Transcriptional activation of chlorocatechol degradative genes of *Alcaligenes eutrophus* NH9

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Chlorocatechol degradation through modified *ortho*-cleavage is a critical catabolic pathway for the utilization of many chloroaromatics by *Pseudomonas* and *Alcaligenes* species. Recently, a gene cluster was isolated from *Alcaligenes eutrophus* NH9 which allows this strain to utilize 3-chlorobenzoate via 3-chlorocatechol as the sole source of carbon and energy. This ca. 6kb gene cluster consists of five genes: the *cbnABCD* operon which encodes the chlorocatechol degradative enzymes and the divergently transcribed *cbnR* which encodes the LysR-type transcriptional regulator of the *cbn* operon. The *cbnR-ABCD* gene cluster is nearly identical to the chlorocatechol genes (*cbR-CDEF*) of the 1,2,4-trichlorobenzene degrading bacterium *Pseudomonas* sp. P51. Transcriptional fusion studies were used to demonstrate that *cbnR* was a positive regulator of *cbnABCD* expression. Expression was induced 210-fold when the cells were grown in 3-chlorobenzoate and 570-fold when the cells were grown in benzoate which are catabolized via 3-chlorocatechol and catechol, respectively. In vitro transcription assays were used to confirm that 2-chloro-*cis*-*cis*-muconate (2-CM) and *cis*-*cis*-muconate (CCM), intermediates of the 3-chlorocatechol and catechol pathways respectively, were inducers of this operon. It is particularly interesting that CCM is an inducer for the chlorocatechol pathway because it is presumed not to be degraded completely by the enzymes encoded by *cbn* genes. In related catechol (*catBCA*) and chlorocatechol (*clcABD*) operons of *Pseudomonas putida*, only the intermediates of the regulated pathway, CCM for *catBCA* and 2-CM for *clcABD*, can act as significant inducers. Specific binding of CbnR protein to *cbnA* promoter region was demonstrated by gel shift assays and DNaseI footprinting analysis. A region of ca. 60 bp from -20 to -80 upstream of the *cbnA* transcriptional start point was protected from DNaseI cleavage by CbnR with a region of hypersensitivity to DNase I cleavage clustered at -50. Circular permutation gel shift assays were used to demonstrate that CbnR bent the *cbnA* promoter region to an angle of 78° and that this angle was relaxed to 54° upon the addition of inducer. Similar relaxation of bending angles upon the addition of inducer molecules has been observed with the *catBCA* and the *clcABD* promoters. This may indicate a conserved transcriptional activation mechanism of *ortho*-cleavage pathway genes, while the *cbn* system is distinct because it has retained the recognition of both CCM and 2-CM as inducers.

Recruitment of an alkyl-substituted phenol/benzene regulated system for the evolution of a unit peptide phenol hydroxylase

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We have previously described a novel unit peptide phenol hydroxylase for *Burkholderia pickettii* PKO1 (1) and subsequently focused on its regulation and organization within the toluene-3-monooxygenase pathway (2,3). Results of the present work suggest an unusual evolutionary change for expression of the phenol hydroxylase gene (*thuD*) may have obtained. The gene encoding this unit peptide, in point of fact, is located within DNA sequences homologous to a toluene/benzene-2-monooxygenase previously described by us for a different strain, *Burkholderia* sp. strain JS150 (4).

We have sequenced 3 kb of DNA spanning the region encoding the *thuD* gene product (phenol/cresol hydroxylase). Various regions of this 3-kb DNA fragment were fused to a LacZ expression system to ascertain the location of the *thuD* promoter and the binding site for its regulator, TbuT. The 5' end for transcripts for the putative promoter of *thuD* was also analyzed using primer extension analysis. Collectively, these results revealed that the promoter was located 2.5-kb upstream of the region encoding the *thuD* gene product whose N-terminal region had been previously determined by peptide sequencing. Remarkably, the intervening 2.5-kb region which included *thuD* showed sequence identity to nucleotide sequences we reported previously for a multi-subunit toluene-2-monooxygenase cloned from a different bacterium, *Burkholderia* sp. strain JS150, for which phenols are also substrates and effectors.

When the DNA sequence for *thuD* and its contiguous 2.5-kb upstream region was compared to the entire toluene-2-monooxygenase sequence cloned from strain JS150, a promoter proximal region encoding three reading frames showed 99 percent identity to corresponding proximal subunits for the toluene-2-monooxygenase operon. Within the region corresponding to the strain PKO1 *thuD* gene, however, sequence homology with the strain JS150 toluene-2-monooxygenase was reduced to 64 percent overall identity. We believe that these results suggest that through evolution either the *thuD* gene of strain PKO1 was derived from a 2-monooxygenase-like pathway by deletions and molecular rearrangements, or alternatively, the *thuD* gene recruited a regulatory system which is activated by benzene, alkyl-substituted benzenes and phenols by recombination of the *thuD* structural gene into the toluene-2-monooxygenase pathway.

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Cloning of the styrene monooxygenase gene from the styrene degrading strain *Pseudomonas putida* CA-3.

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Styrene degradation in *P. putida* strain CA-3, has previously been shown to proceed via styrene oxide, phenylacetaldehyde and phenylacetic acid, which is further oxidised to Krebs cycle intermediates (1). The initial step in the pathway is an oxygen-dependent epoxidation of styrene to styrene oxide, which is catalysed by the enzyme styrene monooxygenase (SMO). The ability of *P. putida* CA-3 to degrade styrene and intermediates of styrene degradation is inhibited in the presence of several more readily utilizable carbon sources, such as glutamate and citrate, in batch grown cells (2). In addition catabolite repression of styrene metabolism in strain CA-3 has also been observed, under continuous-culture conditions; when the strain is grown on styrene or phenylacetic acid in the presence of growth-saturating concentrations of succinate or glutamate under sulfate limitation (3). In order to study the regulation of styrene degradation in *P. putida* strain CA-3, PCR primers were designed based on the sequence of the styrene monooxygenase (SMO) gene in *Pseudomonas fluorescens* strain ST and used to clone the SMO gene in CA-3. Sequence analysis confirms that the SMO genes in *P. putida* CA-3 and *P. fluorescens* strain ST are highly homologous.

Data will be presented on the transcriptional regulation of the SMO gene when the strain is cultured under a variety of different physiological conditions.

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Role of the N-terminal region of the Phenol Responsive σ^{54} -Dependant Activator DmpR in Signal Sensing and Inter-Domain Repression.

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The σ^{54} -dependent *Pseudomonas* CF600 derived transcriptional activator DmpR regulates expression of specialised methylphenol catabolic enzymes. The activity of DmpR is itself directly controlled by interaction with aromatic compounds that serve as pathway substrates. Like other σ^{54} -dependent regulators, DmpR acts from a distance and has a distinct domain structure consisting of i) an N-terminal sensing (signal reception) A-domain, ii) a short flexible B-domain that links the A-domain to the C-domain, iii) a central activation C-domain involved in ATP hydrolysis and interaction with the transcriptional apparatus, and iv) a C-terminal DNA binding D-domain.

Previous work using chimeric proteins and effector specificity mutants has demonstrated that the N-terminal A-domain of DmpR mediates the specificity of activation by aromatic effectors. Direct interaction of DmpR with aromatics leads to expression of its otherwise repressed ATPase activity mediated by the central C-domain. Mutant and deletion analysis of DmpR has led to a working model whereby, in the absence of effectors, the A-domain serves to mask the ATPase and transcriptional promoting activity of the C-domain by specific A/C inter-domain interactions. Here we dissect the A/C domain interaction by expressing portions of the wild-type and genetically defined mutants of DmpR as separate epitope tagged polypeptides, and by measuring the ability of the A-domain polypeptide to repress the activity of the C-domain *in vitro* and *in vivo*. *In vivo*, the repressive activity of the A-domain was found to be *trans*-dominant over DmpR, and co-immunoprecipitation experiments showed that the A-domain interacts specifically with the C-domain of DmpR. *In vitro*, the A-domain was found to be competent to repress the ATPase activity of the C-domain and was influenced in a predictable manner by inclusion of genetically defined mutations. Taken together these data suggest that physically uncoupled A-domain acts as an autonomous repressor. However, in all assays the repressive function of uncoupled A-domain was found to be unresponsive to the presence of aromatic effectors. This result suggests that either the A-domain polypeptide is insufficient to bind effectors, or that physical uncoupling of the A-domain does not allow transduction of the effector binding signal. The results of assays to test the ability of DmpR and truncated derivatives to bind radiolabelled phenol will be presented in the light of the mechanistic implications of aromatic non-responsiveness.

135

Molecular characterization of the genes *pcaG* and *pcaH*, encoding protocatechuate 3,4-dioxygenase, which are essential for the vanillin catabolism of *Pseudomonas* sp. HR199.

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The newly isolated eugenol degrading strain *Pseudomonas* sp. HR199 [1] is also able to utilize vanillin as sole carbon source for growth. Both substrates are metabolized via protocatechuate. After nitroguanidine mutagenesis mutants of this strain were obtained, which were unable to grow on vanillin but retained the ability to utilize eugenol or protocatechuate as carbon source for growth. One of these mutants (SK6169) was used as recipient of a *Pseudomonas* sp. HR199 wild type genomic library, constructed in the cosmid pVK100. Complementation was achieved with a 5.9-kbp *EcoRI* fragment (E59). The nucleotide sequence of E59 was determined. The deduced amino acid sequences from three corresponding open reading frames (ORF) revealed high degrees of homology to the genes *pobA*, *pcaG* and *pcaH*, encoding p-hydroxybenzoate hydroxylase and the two subunits of protocatechuate 3,4-dioxygenase, respectively, from different sources [2]. Two additional ORF encoded probably regulatory proteins. One of these exhibited about 77% identity to the PobR protein from *Pseudomonas aeruginosa* and most probably regulates the expression of *pobA* in *Pseudomonas* sp. HR199. The second ORF exhibited about 55% identity to the PcaQ protein from *Agrobacterium tumefaciens*, which represents a transcriptional activator of the LysR family. This ORF most probably regulates the expression of *pcaH* and *pcaG* in *Pseudomonas* sp. HR199. Since mutant SK6169 was also complemented by a subfragment of E59, only harboring *pcaH*, this mutant seems to lack a functional β -subunit of the protocatechuate 3,4-dioxygenase. Two additional mutants (SK6184 and SK6190), which exhibited the same phenotype as mutant SK6169 were not complemented by E59. During growth of these mutants on eugenol or protocatechuate as carbon source, a compound was accumulated in the medium, which was identified as β -carboxy-cis,cis-muconic acid. In conclusion, vanillin seems to be exclusively degraded via the *ortho* cleavage pathway in *Pseudomonas* sp. HR199.

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136

Dimerization domain of dehalogenase IVa of *Pseudomonas cepacia* MBA4

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Pseudomonas cepacia MBA4 can grow on monobromoacetic acid (MBA) as the sole carbon source. It possesses a dehalogenase which catabolizes the MBA. Dehalogenase IVa (Deh IVa) can cleave the halo groups from L-2-haloacids. Deh IVa exists as a homodimer, with each subunit of 25.9 kDa. In the current study, we aim to characterize the amino acid sequences responsible for the protein-protein interaction of the 2 subunits. To study the dimerization domain, deletion of the Deh IVa from the amino-, carboxyl-terminals and internal regions were done. These were achieved by deleting the DNA sequences (*hdl IVa*) encoding for the subunit of the Deh IVa by means of Polymerase Chain Reaction. The constructs containing whole or deleted *hdl IVa* were used for coupled *in vitro* transcription and translation, the proteins produced were electrophoresed on a native gel, and the dimeric ability of the protein was checked. Certain regions were found to be important for the dimerization of the subunits. Fine internal deletion and site-directed mutagenesis are in progress to locate specific amino acids which are responsible for the interaction between the 2 subunits.

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137

Molecular Biology of Styrene Degradation in *Pseudomonas* sp. VLB120 and its Application in Biotechnology

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Pseudomonas sp. VLB120 was isolated from forest soil using styrene as the sole source of carbon and energy. Styrene oxide and phenylacetaldehyde, known intermediates of styrene degradation *via* attack on the vinyl side chain (1), also supported growth of this microorganism. Furthermore, it is highly resistant to solvents and is able to grow on mineral medium with 1 % (v/v) styrene and in LB medium in the presence of 10 % styrene. To exploit the styrene degradation pathway for styrene oxide synthesis, we have cloned and sequenced a 5.7 kb *Xho*I fragment from a VLB120 genomic DNA library. This fragment enabled *Escherichia coli* strains to convert indole into indigo. The nucleotide sequence revealed six open reading frames that seem to be involved in transforming styrene to phenylacetic acid (ORFs 3 to 6) and in regulation (ORFs 1 and 2). The latter gene products are homologous to two component regulatory systems. Deletion analysis of the 5.7 kb *Xho*I fragment indicated that ORFs 3 and 4 encode a styrene monooxygenase that converts styrene to styrene oxide. Homologies on the amino acid level suggest that ORF 3 encodes a monooxygenase that catalyses the epoxidation and that ORF 4 is an electron transfer protein which is similar to members of a newly identified family of NADH:FMN oxidoreductases. The ORF 5 gene product converts styrene oxide to phenylacetaldehyde and has no homologues in the data bases. ORF6 shows homology to two *E. coli* phenylacetaldehyde dehydrogenases. We assume that ORFs 1 and 2 constitute a positive regulator system which controls transcription from a promoter upstream of ORF 3. Taken together, the data suggest that styrene degradation to styrene oxide occurs *via* a two component styrene monooxygenase, a styrene oxide isomerase and a phenylacetaldehyde dehydrogenase to yield phenylacetic acid which is a common carbon source for *Pseudomonas*. *E. coli* recombinants carrying ORFs 1 to 4 were used to determine the enantioselectivity of the biotransformation of styrene to styrene oxide. The reaction is highly enantioselective yielding the S-enantiomer with an enantiomeric excess of at least 99.5 %.

Reference:

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138

Characterization of an arginine regulatory protein, ArgR, and its interactions with
the control regions for certain biosynthetic and catabolic *arg* genes in
Pseudomonas aeruginosa

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An arginine regulatory gene, *argR*, encodes a polypeptide with significant homology to the AraC/XylS family of regulatory proteins. Inactivation of *argR*, by gene replacement employing a gentamycin cassette, abolished arginine control of the biosynthetic enzymes encoded by the *car* and *argF* operons. Furthermore, *argR* inactivation abolished induction of several enzymes of the arginine succinyltransferase pathway. This pathway, encoded by the *aru* operon, is considered the major route for arginine utilization by *P. aeruginosa* under aerobic conditions. Consistent with these results, the *argR::Gm* derivative was unable to utilize arginine or ornithine as a sole carbon source. ArgR was purified to homogeneity and shown to consist of two equal subunits, each with a molecular mass of 37000 Da. DNase I footprinting showed that ArgR protects a region of 45-47 bp that overlaps the promoters for the biosynthetic *car* and *argF* operons, indicating that ArgR exerts its negative control on expression of these operons by steric hindrance. Studies were also carried out with the catabolic *aru* operon; quantitative S1 experiments showed that expression of the first gene in this operon, *aruC*, is initiated from an arginine-inducible promoter. Studies with an *aruC::lacZ* fusion showed that this promoter is under the control of ArgR. DNase I experiments indicated that ArgR protects two 45 bp binding sites upstream of *aruC*; the 3'-terminus for the downstream binding site overlaps the -35 region for the identified promoter. Alignment of ArgR binding sites reveals that the ArgR binding site consists of two half-sites, in a direct repeat arrangement, with the consensus sequence, TGTCGCN₆AAN. Premethylation interference and depurination experiments with the *car* and *argF* operators identify a common sequence, 5'-TGTCGC-3', which is important for ArgR binding.

Degradation of chlorobenzoates by bacteria isolated from the soil
contaminated by PCB in the Czech Republic

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Chlorobenzoates are important environmental intermediates in the microbial degradative pathways of polychlorinated biphenyls (PCBs) and benzoate herbicides. Two PCB contaminated sites in the Czech Republic, the soil at Zamberk and the sediment at Milevsko, were screened for the presence of chlorobenzoate degraders. Sixteen strains degrading chlorobenzoates (CBAs) were isolated from the soil compared to only four CBA degraders isolated from the sediment. From these strains, only four degraders from the soil and one from the sediment, respectively, were shown to carry complete chlorobenzoate pathway and were further characterized. The degradation of CBAs by these isolates proceeded via intradiol cleavage of the aromatic ring. The activities of both catechol 1,2-dioxygenase (EC 1.13.11.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) were detected in crude cell extracts of these strains using catechol as a substrate. A large plasmid was detected in the strain designated A18 and preliminary results indicated the presence of the gene for *ortho*-chlorobenzoate dioxygenase responsible for the conversion of 2,5-dichlorobenzoate and 2-chlorobenzoate to 4-chlorocatechol and catechol, respectively. We have concluded that in comparison of two equally contaminated PCB sites from the same region, the soil has been more beneficial for the development of chlorobenzoate degrading population than the sediment.

POSTERS ABSTRACTS

Number 141-215

Sunday Sept. 7
9.00 am-5.00 pm
&

Monday Sept. 8
9.00 am-1.00 pm

Control of *Pseudomonas aeruginosa* elastase and rhamnolipid biosynthesis genes by the *las* and *rhl* quorum-sensing systems

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The *las* and *rhl* quorum-sensing systems regulate virulence gene expression in *Pseudomonas aeruginosa*. The *las* and *rhl* systems consist of a transcriptional activator (LasR and RhlR, respectively) and a putative autoinducer synthase (LasI and RhlI, respectively). LasI and RhlI direct the synthesis of the autoinducers N-3-oxo-dodecanoyl homoserine lactone (PAI-1) and N-butyryl homoserine lactone (PAI-2), respectively. Induction of *lasB* (encoding elastase) and other virulence genes requires LasR and PAI-1. Rhamnolipid production in *P. aeruginosa* has been reported to require both the *rhl* system and *rhlAB* (encoding a rhamnosyltransferase). We generated a *P. aeruginosa* PAO1 *lasI* mutant and a *lasI*, *rhlI* double mutant. Rhamnolipid production and elastolysis were abolished in the double mutant and were significantly reduced in the *lasI* mutant and a *rhlI* mutant. We were able to complement these mutations by the addition of the wild type genes on plasmids or exogenous autoinducers. Finally, the specificity of the *las* and *rhl* systems was examined in *Escherichia coli* bioassays. The *las* system had a much greater effect on *lasB* than on *rhlA*, and similarly, the *rhl* system preferentially activated *rhlA* over *lasB*. We also found that maximal LasR and RhlR activity required the presence of the cognate autoinducer, PAI-1 or PAI-2 respectively. The results presented here further characterize the roles of the *las* and *rhl* quorum-sensing systems in virulence gene expression.

141

The genes involved in glyphosate utilization by *Pseudomonas pseudomallei* confer tolerance to plants

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Thirty-four strains of *Pseudomonas pseudomallei* isolated from soil were selected for their ability to degrade the phosphonate herbicide glyphosate. All strains tested were able to grow on glyphosate as the only phosphorus source without the addition of aromatic amino acids. One of these strains, *P. pseudomallei* 22, showed 50% glyphosate degradation in 40 h. in glyphosate medium. From a genomic library of this strain constructed in pUC19, we have isolated a plasmid carrying a 3.0-kb DNA fragment which confers to *E. coli* the ability to use glyphosate as a phosphorus source. This 3.0-kb DNA fragment from *P. pseudomallei* contained two open reading frames (*glpA* and *glpB*) which are involved in glyphosate tolerance and in the modification of glyphosate to a substrate of the *Escherichia coli* carbon-phosphorus lyase, *glpA* exhibited a significant homology with the *E. coli* hygromycin phosphotransferase gene. It was also found that the hygromycin phosphotransferase genes from both *P. pseudomallei* and *E. coli* confers tolerance to glyphosate.

Tobacco (cv. *Xanthi*) plants transformed with the hygromycin B phosphotransferase gene were able to grow in culture medium containing glyphosate at 2.0 mM. The growth of *tobacco calli* in media containing increasing glyphosate concentrations was measured. The ID50 for glyphosate was 1.70±0.03 mM for hygromycin-B resistant plants, and 0.45±0.02 mM for control plants. Regenerated plants and progeny selected for resistance to hygromycin B were tested for glyphosate tolerance by spraying them with Faena herbicide (formulated glyphosate with surfactant) at a dose equal to 0.24 kg/ha. This was two times the dose required to kill 100 percent of the control plants. Phosphotransferase activity was measured in the extracts of the transformed leaves by the incorporation of 32P from [γ -32P]ATP and it was observed that hygromycin B phosphotransferase was able to recognize the molecule of glyphosate as substrate.

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142

Acquisition of the phenol-degrading operon *pheBA* by different indigenous *Pseudomonas* species and characterisation of one PHE-plasmid generated during this acquisition

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Horizontal transfer of genes with selective value in environment six years after their introduction into watershed has been observed. Expression of the gene *pheA* encoding phenol monooxygenase which is linked to the *pheBA* operon (1) allows pseudomonads to use phenol as growth substrate. *Pseudomonas putida* strains carrying this operon in plasmid state were used for bioremediation after the fire-accident in the oil shale mine "Estonia" in Estonia in 1988. The water samples used for studying the fate of the genes introduced were collected in 1994. The same gene cluster was redetected in *Pseudomonas* strains, isolated from water samples of a near-by watershed, which has been continuously polluted with phenols due to oil shale industry leachate. Among more frequently existing counterparts of the *dmp*-genes (2), also the *pheA* gene was represented in phenol degrading strains. Area of finding of the *pheA* gene was restricted with regular route of phenolic leachate to the Baltic Sea. Nine *Pseudomonas* strains belonging to four different species (*P. corrugata*, *P. fragi*, *P. stutzeri* and *P. fluorescens* biotypes B, C, F) and harboring horizontally transferred *pheBA* operon were investigated. In these nine *phe*-operons the *phe*-genes were clustered in the same manner and connected with the same promoter as in the case of the original *pheBA* operon. One 10.6 kb size plasmid carrying *pheBA* gene-cluster was sequenced and structure of the plasmid, named pAM10.6, was described.

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Plasmid-state catalase gene from phenol degradative *Pseudomonas* strain: Possible role of this gene for survival of bacteria in toxic wastewater

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We have isolated and characterized a plasmid-born catalase isozyme KatA from the phenol/cresol-degrading *Pseudomonas fluorescens* biotype F isolate Cb36. Catalase as an integral component of the bacterial cell's response to oxidative stress is widely represented in aerobic bacteria. However, the KatA characterized by us is the first isozyme that is found to be encoded by a biodegradation plasmid. The 10.6 kb -long plasmid pAM10.6, that encodes beside phenol-degradation operon *pheBA* (1) also the gene *katA*, is easily transferable by conjugative plasmids or by DNA transformation. The comparison of the deduced aa sequence of the *katA* gene with other so far known isozymes indicates nonpseudomonal origin of the *katA* gene. KatA is distanced notably from isozyme of *P. fluorescens* (2) and also from other known isozymes of Pseudomonads. It reveals similarity with isozymes of *Haemophilus influenzae* (81% overall identity), *Neisseria gonorrhoeae* and *Bacteroides fragilis*. Therefore we expect that this *katA* gene has horizontally transferred into the *P. fluorescens* strain Cb36. Introduction of the *katA*-expressing plasmid pAM10.6 into *Pseudomonas putida* strain PaW85 resulted in approximately 20 time higher catalase activity in transformant than in a wild type strain. Comparison of survival of *P. putida* PaW85 strains carrying derivative of the pAM10.6 with *katA* deletion and original pAM10.6 demonstrated that presence of functional *katA* gene in cell resulted in increased level of resistance of bacteria against several toxic compounds. We presume that increased level of the common oxidative stress response could be with selective value for *katA* gene that has placed this gene to plasmid state for rapid propagation of the *katA* in cell population exposed to toxic compounds in polluted environment. Further experiments to describe gene regulation of *katA* are in progress.

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Monoclonal antibody to *Burkholderia pseudomallei* and its potential for diagnosis of melioidosis

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The IgM producing monoclonal antibody (MAb) specific to 30 kDa protein antigen of *B. pseudomallei* was produced by *in vitro* immunization with crude culture filtrate of *B. pseudomallei*. The MAb was highly specific to *B. pseudomallei* as tested by indirect Enzyme linked immunosorbent assay (ELISA) and immunoblotting using a panel of crude whole cell antigens extracted from *B. pseudomallei*, *B. cepacia*, *P. aeruginosa*, *P. putida*, and *E. coli*. To explore further the specificity of the MAb, direct agglutination of the antibody to the bacteria was performed. The bacteria are 130 *B. pseudomallei* (128 isolated from hemoculture, one isolated from pus and urine), 10 *Salmonella* spp., and one isolated each of *A. anitratus*, *B. cepacia*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *Proteus* sp., *P. aeruginosa*, *P. putida*, *S. aureus*, *Streptococcus* group B, *Streptococcus* group D, and *X. maltophilia*. The MAb reacted with all 128 *B. pseudomallei* isolated from hemoculture. However, it failed to react with others bacteria and the 2 *B. pseudomallei* isolated from pus and urine. The MAb has potential for rapid identification of *B. pseudomallei* in a short time incubated hemoculture. In addition, instead of biochemical identification of the bacterium in primary culture which take about 1-2 days, the antibody can be used to identify the bacterium within 10 minutes. Furthermore, with the combination of the MAb and recombinant DNA technology, large amounts of purified protein can be obtained readily and safely. Once the antigen is purified it can be used as antigen for rapid diagnosis of melioidosis.

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145

Mineralization of Highly Persistent 1,2,3,4-Tetrachlorobenzene by *Pseudomonas chlororaphis* RW71

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Chlorobenzenes represent a class of hazardous compounds used as building blocks for chlorophenol syntheses. Former sites of production as well as industrial waste deposits exhibit high levels of contamination. Here we report on the isolation of a strain capable of mineralizing 1,2,3,4-tetrachlorobenzene from a mixed culture originally obtained from soil samples from a former industrial production plant. The strain used the target compound but not benzene as a sole source of carbon and energy. It was identified upon analysis of the sequence of 16S rDNA as a member of the pseudomonads. Elucidation of the degradative pathway gave indications for an initial attack of the compound by a dioxygenase and subsequent rearomatization of an intermediary dihydrodiol to tetrachlorocatechol which was identified by GC-MS and HPLC. Tetrachlorocatechol as well as 2,3,5-trichlorodienelactone were transformed by crude extract to their corresponding products tetrachloromuconic acid and 2,3,5-trichloromaleyl acetic acid. A catechol 1,2-dioxygenase of an extraordinarily high ratio of 2.6 for 3-chlorocatechol towards catechol was detected in crude cell extracts. A lower chlorocatechol degradative pathway is proposed upon identification of several intermediates by GC-MS as their methyl ester derivatives.

146

Inducibility of the Promoter of *Pseudomonas oleovorans* GPo1 Involved in Synthesis of Poly-3-Hydroxyalkanoates in Continuous Culture

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Poly-3-hydroxyalkanoate (PHA) is a plastic material produced by fluorescent *Pseudomonads* belonging to rRNA homology group I. The synthesis of PHA in *Pseudomonas oleovorans* GPo1 begins at the end of the exponential growth phase in the presence of excess carbon source when nitrogen limitation starts. The *pha* locus of *P. oleovorans* consists of at least four open reading frames which code for the two PHA polymerases (PhaC1 and PhaC2), a PHA depolymerase (PhaZ) and a protein of unknown function (PhaD) (1).

Optimal synthesis of the PHA polymerases in the wild type strain would provide higher flexibility for the PHA production. Recent studies have revealed that PhaC1 polymerase of *P. oleovorans* GPo1 can be detected by Western blot analysis in the wild type strain only when cells are cultured in the presence of octanoate (2). On the basis of these findings, the goal of this work is to study the factors involved in the transcriptional regulation of *phaC1* gene expression in *P. oleovorans* GPo1.

A *lacZ* reporter system containing *phaGpo1::lacZ* fusion has been constructed by PCR technique. These reporter system has been transferred into the chromosome of *P. oleovorans* GPo1 and *E. coli* MC4100 strain (*lacZ*) in order to investigate the expression of the reporter gene in monocopy. The recombinant strain of *P. oleovorans* carrying *lacZ* fusion has been cultured in continuous culture under different growth conditions, and the β -galactosidase activities and PHA production have been determined. The analysis of the data obtained at this point strongly suggests that expression of the *phaC1* gene is regulated in the wild type strain.

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Purification and characterization of HexR, a putative repressor protein involved in the regulation of carbohydrate catabolism by *Pseudomonas aeruginosa* PAO1

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Catabolism of carbohydrates by *Pseudomonas aeruginosa* elicits the induction of a coordinately regulated, multiopertonic group of genes known as the *hex* regulon. The *hex* regulon encodes the Entner-Doudoroff pathway enzymes 6-phosphogluconate dehydratase (Edd) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (Eda), as well as the genes encoding glucokinase (Gik), glucose 6-phosphate dehydrogenase (Zwf) and NAD-specific glyceraldehyde 3-phosphate dehydrogenase (NAD-Gap). Previous work (1) led to the identification of a 247 bp region of chromosomal DNA known as the *hexC* locus. The *hexC* locus contains a 127 bp intergenic region separating the divergent open reading frames of *gap* and *edd* and a portion of the amino-terminal coding region of each gene. The presence of the *hexC* locus on a multi-copy plasmid in cells grown in the absence of carbohydrate inducers results in a 2-9 fold increase in the basal-level activities of the *hex* regulon enzymes. This phenotype strongly suggests the presence of *cis*-acting sequences of the *hexC* locus capable of titrating a negative regulatory protein(s). The *hexC* locus contains multiple dyad symmetry motifs and three consensus integration host factor (IHF) binding sites.

Gel-mobility shift assays demonstrate: 1) purified *Escherichia coli* IHF binds the *hexC* locus and, 2) a *hexC* locus binding activity in crude extracts of *P. aeruginosa* with different mobility than IHF-*hexC* locus complexes. We have utilized heparin agarose chromatography, anion-exchange chromatography, size-exclusion chromatography and DNA-affinity purification to purify >200-fold the protein(s) responsible for the *hexC* effect. This protein, which we have designated as HexR, has an apparent native molecular weight (MW) of ~120 kDa. *P. aeruginosa* IHF has a native MW of ~20 kDa. Consistent with the hypothesis that the *hexC* locus contains a binding site for a repressor, partially purified HexR binds to a probe containing the promoter region of *zwf*. Binding of HexR to both the *hexC* locus and to the *zwf* upstream region is abolished by addition of <100 mM KDPG, suggesting that KDPG is the physiologic inducer of the *hex* regulon genes. Other metabolites of the *hex* regulon enzymes had no such effect. Although purified *E. coli* IHF specifically binds the *hexC* locus, it is unresponsive to the presence of KDPG.

Additionally, *P. aeruginosa* IHF was not co-purified with HexR. HexR likely binds an inverted repeat, (TGTTGTtttACAAACA), centered at -61 nucleotides 5' of the transcriptional start site of *gap*. A similar sequence (TGTTGTtaattACtACA) is located -71 nucleotides 5' of the translational start of *zwf*.

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Comparison of different genes coding *ortho*-chlorobenzoate dioxygenases (*cbd*) in *Pseudomonas* sp. by DNA-DNA hybridization

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PCB biodegradative metabolic pathway is intensively studied problem in many laboratories around the world. The enzymes for its total degradation are coded by two sets of genes. The first one - the "upper" pathway - is located on chromosome and codes the enzymes for PCB degradation leading to the formation of chlorobenzoates. The other set of genes - "lower" pathway - coding the enzymes for biodegradation of chlorobenzoates is carried by plasmids or maybe located on chromosome. These two sets of genes usually occur in different bacteria species.

DNA-DNA hybridization, which demonstrated differences of *ortho*-chlorobenzoate dioxygenases (*cbd*) between strains *Pseudomonas putida* P111 and *Pseudomonas cepacia* 2CBS, was made within the framework of the preparation of the gene library of *ortho*-chlorobenzoate dioxygenase from the strain *Pseudomonas putida* P111. The *cbd* genes of *Pseudomonas cepacia* 2CBS were used as the DNA-probe.

149

Investigation of a nosocomial outbreak of multidrug-resistant *Pseudomonas aeruginosa* in an intensive care unit by different PCR assays and Pulsed Field Gel Electrophoresis.

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Reports about nosocomial infections due to multiresistant *Pseudomonas aeruginosa* are not very frequent but are increasing during the last years. From November to January 1996 different isolates of multiantibiotic-resistant *P. aeruginosa* strains were collected from two patients in the intensive care unit of Hospital de Bilbao (Spain). The purpose of our study was to determine the level of resistance to different antibiotics and to study the epidemiological relatedness of these isolates by enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), arbitrarily primed PCR (AP-PCR), PCR-ribotyping, and pulsed field gel electrophoresis (PFGE).

A total of seventeen strains of *P. aeruginosa* were isolated; eleven from patient-1 and five from patient-2. Susceptibility to several antimicrobial agents were tested by a standardized disk diffusion test and MICs determination according to standard guidelines. The antibiotics tested were ampicillin, ticarcillin, piperacillin, imipenem, meropenem, aztreonam, piperacillin/tazobactam, amoxicillin-clavulanic acid, ceftazolin, cefotaxime, cefturoxime, ceftazidime, ceftriaxone, cefepime, gentamicin, tobramycin, amikacin, chloramphenicol, cotrimoxazol, ciprofloxacin, norfloxacin, ofloxacin. In order to characterize genetically the isolates, different PCR assays were developed. Genomic DNA was obtained by a lysis with proteinase K and Sodium Dodecyl Sulfate. All the isolates were tested separately with the following primers: a) ERIC1(5'-GTGAATCCCGAGGAGGTTACAT-3'); b) AP3 (5'-TCACGATGCA-3'); c) rRNA1 (5'-TTGTACACACCGCCGTC-3') and rRNA2 (5'-GGTACCTTAGATGTTTCAGTTC-3'). The isolates were also analyzed by *Xba*I and *Dra*I macrorestriction followed by PFGE.

Significant differences in antibiotic susceptibility patterns were detected from initial to final isolates from each patient by means of increasing level of resistance to cefepime, meropenem, ciprofloxacin, imipenem, piperacillin/tazobactam and ceftazidime. ERIC-PCR and AP-PCR showed different banding profiles in each patient, but all the amplicates from each patients have the same pattern. None significative differences were detected between PCR-ribotyping amplicates that only showed just one band of 775 bp in all samples. The strain types found by PFGE correlated with AP-PCR and ERIC-PCR profiles except for the first isolate of patient-1 which was collected from this patient but two months before and for a different source.

Our results showed that each patient was infected by a different multiresistant *Pseudomonas aeruginosa* strain and there was not a cross-colonized infection. Furthermore, despite their different antibiotic phenotype the patients were chronically infected by the same bacterial strain. We conclude that genetic typing assays are a useful tool for the epidemiological identification of *Pseudomonas aeruginosa* and although PCR assays showed successful levels of discrimination power, according with our results PFGE results more effective.

150

Colonization of the rhizosphere of corn (*Zea mays*) by a bioluminescent *Pseudomonas putida* strain and its competitive advantages on soil isolates

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Pseudomonas putida strain S1B1 carries a mini-Tn5-*luxAB* transposon inserted in the chromosome, so that after exposure to *n*-decyl aldehyde cells become luminescent. The strain otherwise maintained wild-type growth characteristics under laboratory conditions, and kept the ability to colonize unvegetated soils. The luminescent phenotype was used to show that *P. putida* S1B1 colonized the root system of a number of plant species, and established in the rhizosphere at a high cell density.

Autocompetition assays revealed that *P. putida* S1B1 was as competitive as the parental strain in vegetated and unvegetated soils. Competition assays between *P. putida* S1B1 and bacterial strains isolated from the rhizosphere of corn plants were also performed in unvegetated soils and in the corn rhizosphere. In all cases co-inoculation failed to affect the population of *P. putida* S1B1 or the bacterial isolate, in comparison to single inoculation assays.

Genetic analysis of protein-protein interactions between components of the *Pseudomonas aeruginosa* Type II export apparatus

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The virulence of *Pseudomonas aeruginosa* can be attributed to its ability to secrete toxins via the Type II export system. The assembly and function of the export apparatus requires at least twelve proteins XcpP-Z and PilD/XcpA but the interactions and functions of the individual components are unknown. Many of the components are homologues of gene products required for the biogenesis of the Type IV pilus and may similarly be assembled into a pilus-like macromolecular complex.

Here we describe a strategy for the genetic analysis of Xcp protein-protein interactions and report the first genetic evidence of an interaction between two components of the export apparatus, XcpT (inner membrane protein, homologue of the Type IV pilin) and XcpR (cytoplasmic nucleotide-binding protein). We have determined that an *xcpT* temperature-sensitive mutant, is suppressed by a mutation in *xcpR*, which results in a substitution at a single residue in the amino-terminus of XcpR (S84F). The suppressor demonstrates allelic specificity and also results in an alteration of the electrophoretic mobility of the XcpR protein. To further characterize this potential interaction, we have generated amino acid substitutions at position 84 of XcpR to determine which residues are capable of suppressing the *xcpT* mutant. The resultant proteins were also characterized for their electrophoretic mobility and it was determined that this property correlates with the ability to suppress the *xcpT* allele. We are currently characterizing the nature of the XcpR mobility shift in hopes of determining how this property may play a role in the suppression of the *xcpT* mutant. The implications of an interaction between the cytoplasmic XcpR and the extracytoplasmic XcpT will be discussed.

Chromosomal tandem amplification in *Pseudomonas putida* F1 of a 100 kb transferable genetic element carrying the *clc* genes for chlorocatechol degradation

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Horizontal gene transfer of the chlorocatechol degradative genes (*clc*) from *Pseudomonas* sp. strain B13 to *Pseudomonas putida* F1 gives transconjugants the novel ability to utilize mono- and 1,4-dichlorobenzene as sole substrates (CB⁺); this is due to the combined action of enzymes from the toluene and the chlorocatechol pathways. Such transconjugants were detected in direct filter and plate matings between the strains B13 and F1, and in wastewater treatment microcosms to which the parent strains were added. Transfer of the *clc* genes to indigenous bacteria in the microcosms was also observed. It has been assumed that the *clc* genes reside on a plasmid in *Pseudomonas* sp. strain B13, but we were unable to detect any plasmid in strain B13, F1 or in CB⁺-transconjugants. Pulsed field gel electrophoresis analysis of DNAs from B13, F1 and CB⁺-transconjugants indicated that the *clc* gene cluster was present on a 100 kb discrete genetic element (named the *clc*-element). In one such transconjugant, *Pseudomonas putida* RR22, a total of 7-8 chromosomal copies of the *clc*-element were present when the strain was grown on chlorobenzene. The 100 kb element had integrated in two different loci on the chromosome, with tandem amplification of the element preferentially in one locus. After prolonged growth on non-selective medium the strain RR22 gradually differentiated into subpopulations with lower copy numbers of the *clc*-element. Two separate single copies of the element always remained after deamplification, and derived strains with only two copies could no longer use chlorobenzene as sole substrate. This suggests that the presence of multiple copies of the *clc* gene cluster was a prerequisite for growth on chlorobenzene by *Pseudomonas putida* RR22 and that amplification of the *clc*-element was strongly selected for in the presence of chlorobenzene.

Complementation of mutants from *Pseudomonas putida* KT2440 unable to accumulate PHA from gluconate: Overexpression of *phaG* and purification of its gene product

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Pseudomonads belonging to the rRNA homology group I are able to synthesize polyhydroxyalkanoic acids (PHA) with 3-hydroxydecanoic acid as main constituent from gluconate or other structurally nonrelated substrates involving the fatty acid de novo synthesis pathway. Recently, we identified a genomic 3 kb *EcoRI* fragment putatively encoding the missing link between de novo fatty acid and PHA biosynthesis and identified mutants defective in this step. To identify the complementing unit of this *EcoRI* fragment, which comprised three open reading frames and is capable of complementing mutant NK2.1 from *P. putida* KT2440 that is not able to accumulate PHA grown on gluconate, we did further subcloning into broad host range vector pUCP26. A 1.3 kb *BamHI/HindIII* fragment, which comprised only the entire open reading frame of *phaG*, was inserted into the corresponding sites of vector pUCP26 and the resulting plasmid pBHR75 mediated PHA accumulation in *P. putida* mutant NK2.1 grown on gluconate. In order to obtain biochemical and enzymatic data about *PhaG*, we established the heterologous expression of *phaG* in *E. coli*. To facilitate purification of *PhaG*, we constructed a plasmid expressing a C terminal his(6)-tag fusion protein of *PhaG*. For this purpose, we amplified the *phaG* coding region, introducing the restriction sites *NcoI* and *BamHI* at the N terminus and C terminus, respectively. The PCR product was inserted into the restriction sites *NcoI* and *BamHI* of vector pQE60 (Qiagen). The resulting plasmid pBHR-QG enabled overexpression of *phaG* under *lac* promoter control in *E. coli* JM109. Cell lysates of *phaG* expressing *E. coli* cells were applied to immobilized metal ion affinity chromatography and the *PhaG*-his(6) fusion protein could be purified to homogeneity in SDS-polyacrylamide gels.

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Pseudomonas mainly synthesize medium chain length poly-3-hydroxyalkanoates (mcl-PHAs) polymers, formed of monomers of 6 to 12 carbon atoms. Mcl-PHAs are attracting more and more attention due to their plastic material properties. So far, three genes involved in PHA synthesis in *P. oleovorans* GP01 have been cloned: *phaC1* and *phaC2* encoding PHA polymerases 1 and 2, and *phaZ* encoding PHA depolymerase. Since *E. coli* is unable to produce any kind of PHAs, it is useful to study the PHA biosynthetic pathways in this host. Fatty acid β -oxidation pathway has been postulated to be involved in mcl-PHAs synthesis in *Pseudomonas*, therefore, different fatty acid degradation deficient *E. coli* mutants were investigated for the ability to produce mcl-PHAs. Among the tested strains, *E. coli fadA/B*, *E. coli fadRfadA* or *E. coli fadRfadB* mutants which are defective in particular steps of the fatty acid β -oxidation pathway allowed production of mcl-PHAs from fatty acids when carrying *phaC2*. The amount of mcl-PHAs produced by the *E. coli* recombinants reached over 10% of total cell dry weight (w/w).

Furthermore, different *phaC* expression systems were applied to reach higher PHA concentrations. The *Palk* promoter, which is derived from *P. oleovorans* and can be expressed efficiently in both *Pseudomonas* and *E. coli*, was used for expression of *phaC1*. By using the *Palk-phaC1* expression system, we could reach a PHA content up to 35% of cell dry mass in *E. coli fadRfadA* and *fadRfadB* recombinants. The *Plac* promoter was used for expression of *phaC2*. Application of this system in *E. coli fadRfadA* and *fadRfadB* resulted in PHA production up to 20% of cell dry mass. From the above results, we conclude that no additional gene except *phaC* gene(s) is essential for mcl-PHA production in *E. coli*; and both polymerases of *P. oleovorans* enable PHA production in *E. coli*.

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Three different extradiol dioxygenases were cloned from the naphthalenesulfonate-degrading strain *Shingomonas* sp. BN6. One of these enzymes is according to its amino acid sequence and small size only distantly related to other extradiol dioxygenases. The enzyme oxidized 2,3-dihydroxybiphenyl with the highest specific activities from various substrates tested and was therefore called 2,3-dihydroxybiphenyl dioxygenase (BphC). Surprisingly, the enzyme also oxidized 3-chlorocatechol, which is generally believed to act as a strong inhibitor of extradiol dioxygenases (1). The reaction product of 3-chlorocatechol showed an absorption maximum at 378 nm, which suggested a distal (1,6-) extradiol cleavage of 3-chlorocatechol to a chlorinated muconic acid semialdehyde (2). Because a distal cleavage of a substituted catechol is a rather unusual reaction, we isolated and characterized the product of the reaction to prove the proposed ring-fission reaction.

The ring-fission product was purified, treated with diazomethane and the resulting methylated product analyzed by mass spectroscopy and ¹H-NMR spectroscopy. The results obtained were in accordance with a chlorinated muconic acid semialdehyde. To further differentiate between a proximal or a distal fission mechanism, the ring cleavage product was converted by the addition of NH₄Cl to the corresponding picolinate. The results obtained by ¹H- and ¹³C-NMR spectroscopy indicated the structure of 3-chloropicolinic acid. Because this compound can only be formed from 2-hydroxy-3-chloromuconic acid semialdehyde, a distal ring-fission mechanism was proven.

Surprisingly, the enzyme only oxidized 3-chlorocatechol by a 1,6-ring-fission mechanism. With 3-methylcatechol or 2,3-dihydroxybiphenyl as substrates, only the well-known products resulting from a proximal ring-fission were observed. To get some more information about the reasons for the different ring-fission directions with 3-chlorocatechol or 3-methylcatechol, the reactions with both substrates were compared and some mutant enzymes from BphC were obtained.

Preliminary results with cell extracts from *Pseudomonas putida* mt-2 suggest that the 2-hydroxymuconic acid semialdehyde hydrolase from the TOL plasmid encoded pathway does also convert 3-chloromuconic acid semialdehyde, but only with low specific activities.

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Regulation of genes involved in the heme biosynthesis of *Pseudomonas aeruginosa* dependent on environmental conditions

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The soil bacterium *P. aeruginosa* forms different amounts of tetrapyrroles in dependence of the employed growth conditions. Regulation of heme biosynthesis occurs at the level of 5-aminolevulinic acid formation and the oxidative decarboxylation of coproporphyrinogen III. The expression of *hemA* encoding glutamyl-tRNA-reductase was found dependent on the extracellular oxygen tension and nitrate concentration and intracellular heme levels. The role of Anr, AlgU and a novel nitrate regulatory system in *hemA* transcription will be described. The anaerobic expression of *hemF* and *hemN*, encoding an oxygen-dependent coproporphyrinogenIII oxidase (HemF) and oxygen-independent dehydrogenase (HemN) were strongly induced by the presence of Anr. Surprisingly, the aerobic *hemN* transcription also showed clear Anr-dependence indicating an aerobic function for the transcriptional regulator. The overall adaptation process of heme biosynthesis via differential expression of various *hem* genes mediated by a set of general transcriptional regulator will be discussed.

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157

Function of polysaccharides (EPS and LPS) from *Pseudomonas syringae* pathovars in pathogenesis

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Green leaves comprise the largest fraction of living organic substances covering the earth. Although leaves can be diseased by a vast and yet unknown number of different fungal species, leaf spot causing bacteria belong nearly exclusively to the genera *Pseudomonas* and *Xanthomonas*. The specific capability of these bacteria to colonize the dry plant surfaces of the intercellular space is attributed to specific features of the components of the extracellular slime, i.e. in case of *P. syringae*: alginate, levan, lipopolysaccharides and a few proteins.

Quantitative determination of exopolysaccharides (EPS) produced by *P. syringae* pv. *phaseolicola* in bean leaves showed, that alginate was produced in higher amounts (about tenfold) than levan, and that the bacteria needed 10-fold more EPS to colonize the resistant plant compared to the susceptible one. Rheological studies indicated that lipopolysaccharides (LPS) may participate in determining the narrow host specificity observed for many *P. syringae* pathovars by specifically interacting with plant polymers (pectic polysaccharides). Only when the components originated from 'compatible' mixtures, interactions were characterized by increased viscosity and yield stress resulting in a viscous gelly solution. These results were obtained with different races and strains of the *P. syringae* pathovars *phaseolicola*, *tomato*, *glycinea*, *coriandricola* and *syringae*.

It is assumed that EPS and synergistically interacting macromolecules from bacteria and plants act like a protective shield around bacterial cells in planta, so that moisture is retained and microcolonies are protected from desiccation as well as from bacteriostatic compounds (phytoalexins) and stress molecules (oxygen radicals). Prevention of close contact of bacterial cells with plant cell walls may block recognition and triggering of the hypersensitive reaction.

158

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During an intensive screening programme approximately twenty bacterial strains, able to transform nitrobenzene and its chlorinated derivatives were isolated from the soil. The bacterium which was most active in the transformation of nitrobenzenes was identified as a *Pseudomonas* sp. and selected for this study. The *Pseudomonas* sp. was cultured aerobically and anaerobically in a Czapek-Dox medium.

After one week of incubation of *Pseudomonas* sp. in culture medium amended with 20 ppm ^{14}C -ring-labeled 4-chloronitrobenzene, under aerobic and anaerobic conditions, only small amount of radioactivity disappeared. However, it was possible to find by TLC method, formation of several radioactive metabolites, namely 4-chloroaniline, 4-chloroacetanilid and 4-chloropropionanilid were identified as main transformation metabolites under aerobic conditions. When isolated strain of bacteria was cultivated under anaerobic conditions, additionally 1,3-bis (p-chlorophenyl) triazene was found as a transformative product of 4-chloronitrobenzene. This identification was confirmed by melting point, UV, IR and mass-spectra analysis of isolated metabolites and chemically synthesized corresponding compounds. Reduction to corresponding anilines and subsequent acylation seems to be the main transformative pathway during transformation of mono- and dichlorinated nitrobenzenes in *Pseudomonas* culture medium. 1,3-bis (p-chlorophenyl) triazene was probably formed during chemical reaction nitrites with anilines under anaerobic conditions in arid environment.

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As part of ongoing work examining the bacterial degradation of phenylpropenoids structurally related to lignin, we studied several bacterial strains able to utilise cinnamic acid as carbon source. One of these strains, *P. fluorescens* ST, which was originally isolated for its ability to degrade styrene, is able to grow on cinnamic and 3-phenylpropionic acids as sole carbon and energy sources and employs a *meta*-cleavage pathway for the utilization of these substrates. The genes (*cin*) encoding for the enzymes (cinnamic dioxygenase and cinnamic *cis*-dihydrodiol dehydrogenase) catalyzing the first catabolic reactions had been cloned on a 25 Kb fragment of genomic DNA using the cosmid vector pLAFR3. The position of the genes on the cloned fragment was determined by subcloning. The nucleotide sequence of a 6 Kb fragment containing part of the *cin* operon was determined. By computer analysis five open reading frames (ORF) were found from this sequenced fragment. Functions of the ORFs were assigned based on the amino acid sequence homology to other proteins of known function. From this comparison cinnamic dioxygenase (*CIN* dox) was found to be a multicomponent enzyme containing a ferredoxin (ORF1), terminal oxygenase α (ORF2) and β (ORF3) subunits and a reductase. However, no polypeptide corresponding to the reductase was expressed from the 6 Kb fragment indicating that the corresponding gene is not closely linked to the other *CIN* dox genes. ORF4 codes for the cinnamic *cis*-dihydrodiol dehydrogenase. ORF 5 has homology to known hydratases: TodJ (57%), XylJ (49%) and DmpH (37%).

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Pseudomonas cepacia MBA4 expressed dehalogenase IVa in batch and continuous cultures where as dehalogenase III and other dehalogenases were expressed only in continuous culture. The gene encoding for dehalogenase IVa has been cloned and sequenced. The gene for any of the cryptic dehalogenases has, however not been studied. Here, we report the cloning of a cryptic dehalogenase gene of *P.cepacia* MBA4. A genomic library of *P.cepacia* MBA4 was constructed on a cosmid vector, supercosI and screening of 1200 transfectants yielded five positive clones. Four of these clones were found to produce dehalogenase IVa while only one clone expressed a dehalogenase designated Deh C, migrating differently from that of dehalogenase III and dehalogenase IVa on activity - stained polyacrylamide gel electrophoresis. Characterisation of the structural gene of Deh C will be discussed.

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The potential of utilising plant-microbe systems for *in situ* remediation of contaminated soil is based on the higher microbial activity in the rhizosphere. However, degradation of xenobiotics in the rhizosphere may still be limited by the rate at which indigenous microflora are able to adapt and evolve new degradative activities. On the other hand, introduced degraders often do not perform well. Gene transfer from introduced strains into the indigenous microflora may be one solution to the problem. We studied the transfer of TOL catabolic genes into microbial population of Scots pine mycorrhizosphere. Seedlings of *Pinus sylvestris* pre-infected with a mycorrhizal fungus, *Suillus bovinus*, were grown in two-dimensional microcosms containing pine forest humus. The mycorrhizosphere was inoculated with 5×10^5 cfu of strain *Pseudomonas fluorescens* OS81(pWW0::Km), harbouring the self-transmissible TOL catabolic plasmid pWW0::Km. *m*-Toluene in water-solution (0.05, 0.1 or 0.5 %) was weekly supplied to the microcosms.

After four weeks of regular treatment with 0.5 % *m*-toluate solution there were no fungal hyphae left in the microcosms. Only minor harmful effects of the *m*-toluate were seen on the plants. After 12 weeks, indigenous transconjugants were isolated at the amounts 4×10^4 - 2×10^6 cfu/g of fresh soil or root tip weight. Approximately 10 % of the Tol⁺ bacteria represented the original strain OS81(pWW0::Km). According to API test, isolated indigenous transconjugants were similar to *Pseudomonas chlororaphis* and *Burkholderia cepacia*. They all carried the original TOL plasmid pWW0::Km. However, additional insertions into the plasmid were detected in some of the transconjugants.

In the microcosms which were treated with 0.05 and 0.1 % solutions of *m*-toluate the original inoculated bacteria were detected in the soil and fungal hyphae at concentrations of 10^4 - 10^5 cfu/g of soil or fungal material at four and six weeks after inoculation. After 12 weeks, the number of Tol⁺ cells decreased below detection limits in microcosms treated with the 0.05 % solution. In those treated with 0.1 %, approx. 10^3 Tol⁺ cfu/g of soil were isolated. Some indigenous transconjugants were also detected.

According to these results enrichment of indigenous mycorrhizosphere bacteria which had acquired the TOL plasmid pWW0::Km from an introduced donor strain depended on exogenously applied *m*-toluate. However, possible protective effects of Tol⁺ bacteria on fungal and plant growth was not sufficient in microcosms contaminated with high concentrations of *m*-toluate.

Relationship between antibiotic production and adaptation to salt stress in *Pseudomonas fluorescens* Pf-5

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Pseudomonas fluorescens Pf-5, a biocontrol agent of several soilborne plant diseases, produces at least three antibiotics including pyoluteorin, pyrrolnitrin, 2,4 diacetylphloroglucinol (1,2). Antibiotic production depends on different global regulatory systems including the stationary-phase sigma ss also involved in several stress resistance (3). Osmotic stress, often encountered in rhizosphere, is studied here. When a high osmotic potential is applied through a high NaCl concentration, cell growth and all antibiotic production were highly reduced in rich medium.

The study of osmoadaptation involvement needed the use of media without osmoprotectant that allowed the production of one or several. In these media, the addition of the osmoprotectant glycine betaine restored cell growth in the presence of high salt concentrations. Pyoluteorin production, highly inhibited in the presence of NaCl, is partially restored during exponential growth when glycine betaine is added. Such restoration was not observed for pyrrolnitrin and 2,4 diacetylphloroglucinol productions.

Different antibiotic production kinetics suggested that negative regulation of pyoluteorin production achieved by sigma factor ss is reinforced by osmotic stress and is released in the presence of glycine betaine. Some osmoprotection and osmosensitive mutants are now assessed for their biocontrol activity and their survival in plant rhizosphere. This study outlines the relationship between antibiotic production and osmoadaptation of rhizobacteria and the possible influence of osmoprotectants that could be encountered in rhizosphere.

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New Ethylene-Producing Bacteria, *Pseudomonas syringae* pv. *cannabina* and pv. *sesami*, detected by PCR-Based Amplification of Genes for the Ethylene-Forming Enzyme

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Ethylene is a plant hormone produced by various plants, that is involved in the regulation of numerous physiological processes in plants. Ethylene is also produced by a limited number of microorganisms including plant pathogenic *Pseudomonas syringae* pv. *phaseolicola* (kudzu-strains) (1) and *P. syringae* pv. *glycinea* (2). To detect other ethylene-producing bacteria, a total of about 150 strains including 43 pathovars of *P. syringae* were examined for the presence of ethylene-forming enzyme (EFE) genes by PCR assay. Two sets of primers, which were designed based on sequence data of the EFE gene of *P. syringae* pv. *phaseolicola* PK2 previously reported (3), were used for the amplification of the EFE genes. Besides *P. syringae* pv. *phaseolicola* and pv. *glycinea*, several strains of *P. syringae* pv. *sesami* (a pathogen of *Sesamum indicum*) and pv. *cannabina* (a pathogen of *Cannabis sativa*) generated PCR products with a predicted size. While, any other bacterial strains including strains of plants- and insects origin did not generate the PCR products. A probe of the EFE gene (4) from PK2 strain hybridized to these PCR products, indicating that they originated from EFE genes. PCR-RFLP analyses of PCR products strongly suggested that four pathovars harbor the same type of EFE genes. Furthermore, Southern blot analysis using the probe revealed that an indigenous plasmid (approximate 110 kd) of *P. syringae* pv. *cannabina* carries EFE genes like the plasmids of *P. syringae* pv. *phaseolicola* and pv. *glycinea*. Then, the ethylene production of PCR-positive bacteria was examined by gas chromatography analysis. The strains of *P. syringae* pv. *sesami* and pv. *cannabina* produced ethylene in King's B medium at a level similar to that of the two known pathovars. Thus, two efficient ethylene-producing bacteria were newly detected by the PCR assay.

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Fenitrothion (MEP), carbaryl (NAC) and dinitroaniline-group herbicides including trifluralin and pendimethalin have been widely used in Japan to control pests or weeds. A role of pseudomonads in the microbial degradation of these chemicals in soil was investigated. Bacteria were isolated from farmland and forest soils treated with these chemicals. To isolate selectively the aimed bacteria, the inorganic liquid medium supplemented with the chemicals was used. Several species of the herbicides-degrading bacteria were obtained from the farmland soil. These bacteria were identified as *Alcaligenes* sp. and *Moraxella* sp. degrading trifluralin, and *Alcaligenes* sp. and *Bacillus* spp. degrading pendimethalin. Any pseudomonads degrading the herbicides, however, did not detected. From the forest soil, several MEP-degrading bacteria such as *Bacillus* sp. and *Pseudomonas fluorescens*, and NAC-degrading bacteria such as *Arthrobacter* spp. and unidentified bacteria (not pseudomonads) were isolated. To evaluate the ability to degrade the chemicals of these bacteria, the residue of the chemicals in the medium or soil inoculated with the bacteria was analyzed by gas chromatography (GC). Furthermore, the resultant major metabolites were identified by GC-mass spectrometry. In microbial degradation of MEP, the ability of *P. fluorescens* strains was much weaker than that of strains of *Bacillus* sp. Similar results were obtained in the MEP-degradation tests using stock cultures of *Pseudomonas* spp. and *Bacillus* spp. Only one strain of *P. fluorescens* exhibited a weak ability to degrade it, while *Bacillus pumilus*, *B. subtilis* and *B. cereus* strongly degraded MEP. These results suggest that pseudomonads do not participated greatly in the degradation of MEP, NAC, trifluralin and pendimethalin in farmland and forest soil.

The ability to degrade extracellular polyhydroxyalkanoates (PHA) is far distributed among microorganisms. Degradation is initiated by the secretion of specific PHA-depolymerases (recently reviewed in [1]). In contrast to the utilization of poly(3-hydroxybutyrate) (PHB) the knowledge on the degradation of polyesters consisting of medium-chain-length monomers (PHAMCL) such as poly(3-hydroxyoctanoate) (PHO) is poor. The PHO depolymerase of *Pseudomonas fluorescens* GK13 is the only PHAMCL depolymerase analyzed in detail [2].

Biochemical analysis of the PHO depolymerase of *P. fluorescens* was impaired because of low yields and partial inactivation of the enzyme during the purification procedure. These difficulties were overcome by the observation that the depolymerase specifically binds to purified PHB granules. The PHO depolymerase can be now purified by (i) binding of the protein from the culture fluid to PHB granules, (ii) resolubilization of the depolymerase from centrifuged PHB-granules in 50% 2-propanol, and (iii) gel filtration (or isoelectric focussing).

Mutational analysis of the cloned PHO depolymerase gene (*phaZ*) showed that Ser139, Asp195 and His227 apparently constitute a catalytic triad similar to those of lipases and other serine hydrolases (3, 4). Additional residues of the PHO-depolymerase, which are involved in substrate-recognition and substrate-binding, were identified by PCR-induced random mutagenesis of the structural gene and screening for mutants which were impaired in PHO depolymerase activity but which were not affected in hydrolysis of artificial water-soluble esters. Two phenotypes of mutants were obtained: class I mutants showed reduced PHO depolymerase activity but were still able to hydrolyze the polymer completely to a mixture of monomers and dimers; class II mutants were also reduced in PHO depolymerase activity but were not able to hydrolyse PHO completely. Several hot spots of point mutations were identified after sequence analysis of 30 independently isolated mutants. These hot spots are located in the neighborhood of Ser17 (7 mutants, class II phenotype), Phe50 (4 mutants), Phe63 (5 mutants) or near the catalytic triad amino acids (all class I phenotype). The effect of mutation on PHO depolymerase activity will be shown in detail.

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Analysis of plasmid involvement in virulence in *Pseudomonas syringae* pv. *tomato* strain PT23

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Pseudomonas syringae pv. *tomato* PT23 contains four native plasmids: pPT23A (100 kb) carries the gene cluster for the synthesis of phytotoxin coronatine, which is required for the production of full disease symptoms in tomato plants (1); pPT23B (83 kb) contains a virulence gene D (*avrD*); pPT23C (65 kb) is cryptic and pPT23D (36 kb) is involved in resistance to copper. We have obtained evidence for plasmid involvement in virulence in *Pseudomonas syringae* pv. *tomato* PT23 analyzing the existence of phenotypic changes *in vitro* and *in planta* of PT23 derivative strain WABCD2 (W2; 2) cured of all four native plasmids. Plasmid pPT23B (*avrD*::Tn5) (pB::Tn5, 3) and pPT23A::Sm (pA::Sm, constructed introducing by marker exchange a Sm/Sp cassette) were introduced into W2 by electroporation. The *in planta* populations and the size and number of necrotic lesions produced by strain W2 containing both plasmids A and B (W2AB) did not differ significantly from the wild type values. The strain W2 containing only one of the two plasmids (W2A or W2B) were not able to obtain the size of wild type necrotic lesions. Fifteen clones obtained from a PT23 gene library screened using pA::Sm as a DNA probe were mobilised into the W2B strain. Seven of them are able to increase W2B phenotype *in planta*, and only two contain fragments that are present in pSAY1 (4), a cosmid that includes the gene cluster of coronatine from pPT23A. The other five clones have overlapped DNA fragments that contain 30 kb region from pPT23A.

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Mosaic organization and compatibility of highly related native plasmids in *Pseudomonas syringae* strains

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Strain PT23 of *P. syringae* pv. *tomato* contains four native plasmids, designated A, B, C, and D. Plasmids A and B probably originated from a duplication event, since they share a large amount of DNA, including replication sequences (1). Using the defined minimal origin of replication (cloned in pAKC) from pPT23A as a probe, it was shown that it is highly conserved in different pathovars of *P. syringae* (1), and that as many as five different native plasmids with closely related origins of replication coexist in the same cell. Since many of these plasmids carry genes related to virulence or resistance to pesticides, we were interested in the mechanisms that allow for their stable coexistence. pAKC was transformed by electroporation in ten *P. syringae* strains that contained plasmids related to pPT23A. In PT23 and *Ps* pv. *glycinia* race 4, we observed the specific curing of at least one plasmids, while the remaining strains did not show any alteration of their plasmid profiles. pAKC contains at least three different regions of incompatibility (2), designated as IncA, IncB (origin of replication), and IncC. The observed lack of incompatibility suggest, therefore, that plasmids related to pPT23A must have suffered changes in the three Inc regions that are responsible for this behaviour.

Hybridization experiments using the three Inc regions plus additional DNA adjacent to *oriV*-pPT23A, showed that plasmids related to pPT23A display a mosaic organization of their replication regions. The implications of these results for plasmid evolution and maintenance will be discussed.

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Development of Bioassays to Monitor Phenazine Production and N-acyl-L-homoserine lactones by Pseudomonads in the Rhizosphere.

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Root-colonising fluorescent pseudomonads have been extensively studied for their potential as biological control agents against soilborne plant pathogens. For *Pseudomonas aureofaciens* PGS12 and *P. fluorescens* 2-79, production of the antibiotic phenazine-1-carboxylic acid (PCA) is a major component of the mechanism involved in suppression of take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* and damping off of many crops caused by *Pythium*, *Fusarium* and *Rhizoctonia* spp.. The phenazine biosynthetic locus has been cloned, sequenced and the regulatory genes identified. The transcriptional activator PhzR, acts in concert with the autoinducer N-3-oxohexanoyl-homoserine lactone (OHHL), synthesised by PhzI, to activate the transcription of the phenazine gene cluster. OHHL enables the bacteria to positively regulate transcription of the antibiotic in a cell-density dependent manner. The aim of this research project is to investigate the extent to which N-acyl-homoserine lactones (AHLs) influence phenazine production in the rhizosphere. For this study, a set of bioassays for phenazine and AHLs monitoring have been developed as well as several *lux*-marked and mutant strains. Details of this work will be presented in this poster. The bioassays will also provide the basis to study root and soil influences on the effect of AHLs as signals for inter- and intra-population communication.

169

Nucleotide sequence analysis of the *car* (carbazole) locus from *Pseudomonas* sp. strain CB3.

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The azarene carbazole has been detected as a contaminant of soil and groundwater. Despite toxicity fears the fate of azarenes in the environment has received little attention. Bacterial degradation of aromatic compounds is an important route for the removal of these compounds from the environment. *Pseudomonas* sp. strain CB3 is able to utilize carbazole as sole carbon and nitrogen source [1], and was isolated from a site contaminated with polycyclic aromatic hydrocarbons (PAHs) and high molecular weight phenolics at Rotowaro, near Huntly, New Zealand.

The nucleotide sequence of the *carA1A2A3A4BCD* genes, which encode the first four enzymes required for carbazole catabolism by strain CB3, have been elucidated. The functional extradiol dioxygenase gene, and associated genes, were cloned from genomic DNA. Southern analysis indicated that these genes were chromosomally located and not present on the large (>100-kb) plasmid present in CB3.

Seven structural genes were located and identified by DNA sequencing and expression. The *car* locus encodes, by analogy to characterised *bph* loci, four components of a carbazole dioxygenase enzyme system, a carbazole-2,3-dihydro-2,3-diol dehydrogenase, an extradiol (*meta*-cleavage) dioxygenase, and a hydrolase. The gene arrangement of the *car* locus is homologous to that found in some *bph* loci.

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170

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Pseudomonas tolaasii is the cause of brown blotch of cultivated mushroom (1). The bacterium can exist in two stable phenotypic variant forms, a wild form designated 1116S which produces the toxin tolaasin and a variant form designate 1116R which does not produce the toxin. Tolaasin synthesis is controlled by a complex regulatory pathway which is intricately linked to the control of phenotypic variation.

Five regulatory loci in this pathway have been cloned. The master regulator *pheN* encodes a transmembrane transcriptional regulator which has high homology with *lenaA* of *P. syringae* pv. *syringae* and is deduced to be the environmental sensor (2). The cognate response regulator of *pheN*, designated *pheS*, shows high homology to *gacA* of *P. syringae* pv. *syringae*.

Two downstream regulatory loci, *pheP* deduced to encode a sigma factor-regulating expression of protease production and *pheT*, which differentially regulates toxin synthesis, have been identified.

Phenotypic switching from 1116S to 1116R in *P. tolaasii* has been shown to be due to duplication event in *pheN* gene (3). Progress in analysis of the role of the *recA* gene in this phenotypic switch as well as the functional analysis of these regulatory loci will be presented.

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Pseudomonas aeruginosa produces rhamnose-containing glycolipid biosurfactants which have several industrial and environmental applications (1, 2). In liquid cultures two forms of these rhamnolipids are formed: rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (mono-rhamnolipid) and rhamnosyl-rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (di-rhamnolipid). The biosynthesis of these tenso-active molecules was described since 1963 (3) and proceeds by two sequential rhamnosyl-transfer reactions, each catalyzed by a specific rhamnosyltransferase (Rt 1 and Rt 2 respectively) with deoxy-thymidine-diphospho-L-rhamnose (dTDP-L-rhamnose) acting as rhamnosyl donor in both reactions and β -hydroxydecanoyl- β -hydroxydecanoate or mono-rhamnolipid acting as the respective recipients. Genes coding for biosynthesis, regulation and induction of Rt 1 enzyme are organized in the *rhlABRI* gene cluster around minute 38 of the *P. aeruginosa* chromosome (4). Mutations in any of these *rhl* genes cause a rhamnolipid minus phenotype which indicates that mono-rhamnolipid is an obligate precursor of di-rhamnolipid. The genes encoding Rt 2 are yet to be described.

The product of the *algC* gene has phospho-manno-mutase and lipopolysaccharide (LPS) production and it has been reported to participate both in alginate and in rhamnolipid production and found (5). We analyzed the effect of mutations on the *algC* gene on rhamnolipid production and found that neither form was made. The involvement of other alginate genes in rhamnolipid production will also be presented.

We isolated a mutant (IBT8) derived from the *P. aeruginosa* PAO1 strain that did not produced mono-rhamnolipid while still producing di-rhamnolipid and also produced a defective LPS. This mutation was not complemented by the *rhlRI* regulatory genes (6). We have isolated a plasmid referred to as pNH2198 that can complement both the rhamnolipid and LPS defect in IBT8. Thus we concluded that this plasmid contains a novel regulatory region which regulates the expression of both rhamnolipids and LPS.

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The sequence diversity at six loci (*oriC*, *ampC*, *oprI*, *citS*, *flhC*, *pilA*) of 19 *P. aeruginosa* isolates from various environmental and clinical habitats was assessed. The average rate of polymorphism amongst the analysed loci excluding the hypervariable *pilA* is about 1.5 %, but some genes are even more conserved (*oprI*). Characterization of about 60 single nucleotide substitutions shows a striking overrepresentation of transitions (86 %) and synonymous substitutions (82 %). 89 % of the base substitutions presumably derived from the thermodynamically most stable T•G mismatch followed by A•G and G•G mismatches.

Analysis of the neighbouring 20 bp up- and downstream of each polymorphic site with respect to stacking energies, flexibility of the backbone and hyperfine structure revealed a significant reduction of stacking energies within the 10 bp-environment (one pitch). This gain of thermodynamic stability by the 5 adjacent base pairs might have facilitated the substitution of the central base.

The dinucleotide and trinucleotide frequencies of the genes still retained a non-random distribution even after the codon usage bias had been eliminated. *P. aeruginosa* exhibits a strong bias in its codon usage underlining the 67 % GC-content of the bulk chromosome. The codon adaption indices CAI, a measure of the codon usage bias, of various genes are high (mean of 0.71), in contrast to *E. coli* whose codon adaption indices correlate with the extent of gene expression. The putative high expression of every gene makes *P. aeruginosa* a versatile organism which may adapt easily to new habitats.

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In order to resolve the genetic diversity and structure of natural *P. aeruginosa* populations sequence diversity at six loci (*oriC*, *ampC*, *oprI*, *citS*, *flhC*, *pilA*) of 19 *P. aeruginosa* isolates from various environmental and clinical habitats was assessed. Analysis of nucleotide sequence variation revealed only few highly polymorphic genes, e.g. *pilA* encoding the subunits of type IV-pili. The heterogeneity of the *pilA* locus correlates with the variable genome organization in the pilin gene region. A pilin class-specific sequence insertion leads to the hypothesis that the pilin gene together with its flanking region was acquired from another species.

Despite similar localisation at the bacterial cell surface, the flagellin gene (*flhC*) is much more conserved than the hypervariable *pilA*. Genetic diversity of flagellins is reduced to several nucleotide substitutions and a variable 141-bp central cassette showing 28 % nucleotide and 40 % amino acid diversity.

Except for the highly polymorphic pilin genes sequence diversity in the common gene repertoire does not reflect the diversity of the surrounding genome organization in *P. aeruginosa*. Gene sequences are less polymorphic than the corresponding macrorestriction patterns, leading to the impression that mainly noncoding regions, amplifications, deletions and rearrangements contribute to the substantial diversity of the *P. aeruginosa* chromosome. Molecular evolutionary relationships at each locus which were examined by the neighbour-joining method of tree construction suggest a high frequency of recombination within the *P. aeruginosa* chromosome. An excess of recombination over nucleotide substitution maintains linkage equilibrium between adjacent loci.

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The *mexC-mexD-oprJ* and the *mexA-mexB-oprM* operons encode components of two separate multidrug efflux pumps in *P. aeruginosa* (1, 2). The genes encoding these two pumps were cloned and expressed in an *E. coli* strain deficient in the multidrug efflux pump encoded by the genes *acrA-acrB* (Δ *acrAB*; 3). *E. coli* Δ *acrAB* strains expressing *mexC-mexD-oprJ* or *mexA-mexB-oprM* showed increased resistance to the quinolones, chloramphenicol, erythromycin, SDS, and crystal violet over control strains. An *E. coli* Δ *acrAB* strain expressing *mexA-mexB-oprM* also showed increased resistance to novobiocin and to several β -lactams. These data show that the *P. aeruginosa* multidrug efflux pumps are functional in *E. coli* and that there is no change in their substrate specificities in this heterologous host. In order to characterize these pumps further, experiments that are designed to demonstrate transport of antibiotics into everted vesicles will be performed using these *E. coli* strains. As a complementary strategy to study pump activity, a hexahistidine-tagged *MexB* fusion protein was generated using the pET (Novagen) system. This fusion protein could functionally replace wild-type *MexB* to yield a multidrug resistant phenotype in *P. aeruginosa*. Large quantities of the histidine-tagged *MexB* was purified using nickel-chelating resin. The drug transport properties of this protein will be assayed after reconstitution into synthetic liposomes. These combined approaches of studying *P. aeruginosa* pumps are expected to provide novel insights into the molecular and biochemical events that lead to drug efflux.

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A previously cloned portion of the 66-70 min region of the *P. aeruginosa* chromosome was sequenced and a putative operon of 4 genes, termed *pvc* (pyoverdine chromophore) *ABCD*, identified. Disruption of the *pvc* genes abrogated pyoverdine production in mutant strains consistent with the involvement of this operon in pyoverdine biosynthesis. The products of these genes showed homology to oxygenases (PvcB), hydroxylases (PvcC) and members of the cytochrome c superfamily (PvcD), suggestive of an involvement in the biosynthesis of the pyoverdine chromophore. Downstream of *pvcD* and in the opposite orientation, the previously described *ptxR* gene, encoding a LysR-family transcriptional activator of exotoxin A (1), was identified. A LysR consensus binding sequence was present upstream of *pvcABCD* and deletion of *ptxR* abrogated pyoverdine production and *pvcABCD* expression, indicating that PtxR positively regulates *pvcABCD* expression in *P. aeruginosa*. Still, the cloned *ptxR* gene repressed expression of a *pvc-lacZ* fusion in *E. coli*, suggesting that PtxR possesses both activator and repressor activities.

Expression of *pvcABCD* was negatively regulated by iron and positively regulated by pyoverdine, the latter indicating the pyoverdine (or ferric pyoverdine) upregulates its own expression. A mutant deficient in PvdS, the alternate sigma factor required for pyoverdine production, was deficient in *pvcABCD* gene expression. The deficiency in *pvcABCD* (and pyoverdine) expression in *ptxR* and *pvdS* mutants was overcome by the addition of pyoverdine, indicating that *pvcABCD* expression is regulated at two levels, one responsive to PtxR and PvdS and one independent of these proteins but responsive to pyoverdine. Thus, PtxR and PvdS may function to support initial pyoverdine biosynthesis under iron-limiting conditions, the siderophore (or ferrated siderophore) serving to subsequently upregulate *pvcABCD* via a PtxR-/PvdS-independent mechanism.

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Convergence of Global and Phenol-Responsive DmpR-Mediated Regulation at the σ^{54} -dependent *dmp*-operon promoter

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The *Pseudomonas* sp. strain CF600-derived Po promoter controls the transcription of the fifteen (methyl)phenol catabolic genes of the *dmp*-operon. Specific regulation of the *dmp*-operon is mediated by the divergently transcribed *dmpR* gene product. Appropriate expression of the catabolic enzymes is achieved in the presence of *dmp*-pathway substrates and structural analogues by their direct interaction with DmpR to derepress its transcriptional promoting property and thereby promote transcription from the -24, -12 Po promoter. This aromatic responsiveness is, however, dependent on the physiological status of the cells, for example the growth phase of the culture. These results suggest a second level of regulation dictated by global regulatory factor(s). In this study, we sought to identify the possible players involved in this level of regulation.

To this end, we made use of the luciferase reporter gene to monitor the DmpR-mediated transcriptional activation of Po *in vivo*. Various genetic systems were constructed, both in *P. putida* and *E. coli*, to investigate the involvement of possible global factors and DNA topology in the growth phase dependence of DmpR-mediated phenol-responsive regulation. Genetic dissection of the system allowed us to identify *dmpR*, the Po promoter-upstream region and Po as the minimal *dmp* components required for the global response in *E. coli* and *P. putida*. The regulatory region contains a large inverted repeat encompassing the binding sites for DmpR (UAS1 and UAS2), an IHF consensus binding site overlapping UAS2, and a small inverted repeat between the UASs and Po. Over-expression of DmpR was found to completely abolish the growth phase dependence, suggesting titration of some negatively acting factor(s) by DmpR. Hence, it is likely that the global regulatory signal is processed through the activities of DmpR e.g. i) effector activation, ii) multimerization iii) interaction with σ^{54} -RNA polymerase, or iv) its binding to DNA. We will present the data from experiments on DmpR DNA binding and over-expression of its different functional domains designed to identify the level on which the global regulation acts.

177

The choice of an appropriate selective medium is critical for assessing the genetic diversity of a natural microbial population

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The isolation of microorganisms which are representative of the diversity of a known species can be difficult as a result of inherent bias in the method used. For instance, it has been demonstrated that culture enrichment limits the observed genetic diversity of a microbial species.

The aim of this work was to evaluate the influence of two different isolation media on the assessment of the genetic diversity of a natural population of *Burkholderia cepacia*.

B. cepacia is one of the most promising species in improving crop performance and in controlling several plant diseases. So far, two different selective media, PCAT and TB-T, have been developed for the isolation of bacteria belonging to this species from terrestrial and aquatic environments.

In this study, *B. cepacia* strains were recovered from roots of maize plants grown in a field located at Dragoni, Italy. Plants were randomly harvested from the field, roots were excised and loosely adhering soil was removed. Roots were blended and serial dilutions of these suspensions were plated on PCAT and TB-T media. From each medium 50 *B. cepacia* - like colonies were isolated. On the basis of restriction patterns of 16S rDNA amplified by means of PCR (ARDRA) all the strains isolated from TB-T medium were assigned to the *B. cepacia* species, whereas only 74% of the isolates from PCAT medium were assigned to *B. cepacia* species. Genetic diversity among the PCAT and TB-T isolates was evaluated by random amplified polymorphic DNA (RAPD) technique. The analysis of molecular variance (AMOVA) procedure was applied to determine the variance component for RAPD patterns. Most of the genetic diversity (90.59%) was found within the two groups of isolates, but an appreciable amount (9.41%) still separated the two groups ($P < 0.004$). Mean genetic distances among PCAT isolates and TB-T isolates were 10.39 and 9.36 respectively. Furthermore, variance within PCAT group was higher than that within TB-T group. In conclusion, the two different isolation media select for genetically different *B. cepacia* populations. Moreover, a higher degree of genetic diversity was observed among strains isolated from PCAT medium than from TB-T medium.

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178

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The aerobic respiratory chain of *P. aeruginosa* is composed of at least two major branches. One branch is terminated by a cytochrome c oxidase that is sensitive to micromolar concentration of cyanide and a second branch is terminated by a quinol oxidase that is resistant to millimolar concentration of cyanide.

The *cioAB* genes code for a novel cyanide-insensitive terminal oxidase (CIO) which is related to the cytochrome bd quinol oxidases, but probably does not contain haem d. In addition to lacking CIO activity *cio* mutations have pleiotrophic effects. CIO strains are sensitive to elevated temperatures. Above 37°C the growth of *cio* strains is inhibited and cells are unable to exit stationary phase. These defects are linked to abnormal filamentation of the cells indicating an effect on cell division, most probably in septum formation. We will provide evidence that these pleiotrophic effects of *cioAB* mutation are a consequence of the production of toxic levels of reactive oxygen species.

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Transposition of Tn4652 from chromosome of *Pseudomonas putida* strain PaW85 into plasmid carrying promoterless *pheBA* operon had generated constitutively expressed fusion promoters for transcription of the *pheBA* genes. These promoters consist of terminal sequences of the transposon providing -35 hexamer and sequences of Tn4652 target providing -10 hexamer for the promoter (Nurk et al., 1993). The fusion promoters were formed on the basis of transposon's right and left end. Rate of transcription from the fusion promoters depends on the length of the Tn4652 DNA upstream from the -35 hexamer of the promoters and is growth phase-regulated, being higher in stationary phase cells.

Both termini of Tn4652 contain three IHF-binding consensus sequences. Integration host factor (IHF) is a multi-functional protein of *E. coli*. IHF plays role in transposition of some mobile DNA elements as an accessory factor and it can stimulate or repress transcription (Freundlich et al., 1992). IHF level is 6-10 fold higher in stationary phase cells, than in exponentially growing cells (Ditto et al., 1994).

Transcription from the fusion promoter containing upstream sequences was found to be lower in *P. putida* IHF-deficient strain than in a wild type. Overexpression of genes coding for IHF in *E. coli* affected transcription from both fusion promoters negatively. Gel shift analysis with crude extracts from cells expressing IHF showed that IHF from *E. coli* could specifically bind to the Tn4652 terminal sequences while assay conditions used by us did not allow detect specific binding of IHF from *P. putida*.

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Reporter genes and immunological methods for monitoring inocula activity and persistence in the rhizosphere of a biological control agent.

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Pseudomonas fluorescens 54/96 is an effective biocontrol agent of damping off disease (*Pythium* spp.) in a variety of crop plants. As the mode of action of this biological control agent (BCA) is not fully understood, studies are in progress to determine whether *P. fluorescens* 54/96 protects seedlings by aggressive colonisation or by the production of specific anti-fungal compounds. To allow identification of possible biofunctional genes/operons, several *in vitro* assays have been developed to isolate BCA strains from a library of functional mutants. To develop a better understanding of the ecology and activity of microbial-plant interactions, promoterless reporter genes, *lacZY* (β -galactosidase, lactose permease) have been constructed in pTT5KZY a transposon based mini-Tn5 system (de Lorenzo, V & Timmis, K.N. 1994). Bacterial promoters, induced during colonisation of the rhizosphere and in association with *Pythium* or soil, can be characterised to identify specific molecular interactions between pathogen, BCA and host plant. By raising polyclonal antibodies to *lacY* novel fluorescent microscopic detection assays have been developed to detect the induced expression of reporter genes. This approach has allowed modified inocula to be used to better comprehend the spatial and temporal distribution of colonising bacteria, their persistence in the rhizosphere and improve our understanding of microbial community succession. This reporter probe identifies perception of specific rhizosphere signals that may be relevant in the improvement of BCA activity by cellular signalling therefore by utilising environmentally induced promoters to control the expression of introduced biofunctional genes.

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181

Introduction of fluorescent marker into the chlorobenzoate degrader *Pseudomonas putida* P111

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Genetically engineered bacteria with improved degradation abilities and well equipped naturally isolated degraders may be useful for the bioremediation of contaminated soils and sediments. The stability of nonindigenous strains is usually quite low due to the competition with adapted indigenous population, however, the functional catabolic pathway may be spread into this population by conjugation on transmissible plasmids. Chlorobenzoate degrader *Pseudomonas putida* P111 contains major part of the catabolic pathway for the degradation of *ortho*-chlorobenzoates on a transmissible plasmid pPB111. Genes for the expression of green fluorescent protein (GFP) were subcloned from the plasmid pJBA27 into the transposon cassette of suicide vector pUT which carries modified mini-Tn5 transposon located outside of the inverted repeats (1). This construct was then delivered into the strain *Pseudomonas putida* P111. The successful transfer of GFP operon was confirmed by the growth of recombinant strain P111gfp on mineral salt medium with 2,5-dichlorobenzoate as the only source of carbon and energy and by monitoring characteristic green light emitted at 509nm. Further analysis enabled the isolation of recombinants containing GFP operon on both plasmid and chromosome of the strain *P. putida* P111gfp.

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182

Characterisation of the structural gene of *Pseudomonas aeruginosa* CysB, which and its effect on *algD* transcription.

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Pseudomonas aeruginosa strains infecting patients with cystic fibrosis (CF) acquire a mucoid phenotype due to overproduction of alginate. The key enzyme in alginate synthesis is *algD*, whose promoter is transcriptionally active in mucoid strains and under the control of several *trans*-acting factors, including the integration host factor (IHF). The *algD* promoter (*palgD*) contains two IHF-binding sites (*ihf1* and *ihf2*). Study of IHF binding to *ihf2* of *palgD*, by electrophoretic mobility shift assays, led to the discovery of a protein of 36 kDa (p36) able to bind downstream from *ihf2*, to the 3' region of *palgD*. We isolated the gene encoding this 36 kDa protein. It encodes a protein homologous to the transcriptional factor CysB of *Escherichia coli* and *Salmonella typhimurium*. A CysB-deficient mutant of the mucoid strain CHA was constructed by insertional inactivation of the so called *cysB* gene. We showed that *P. aeruginosa* CysB can act as an activator of *algD* expression (1).

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Thrombin Cleavage Site Mediated Multimerisation of Dehalogenase IVa of *Pseudomonas cepacia* MBA4

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In vitro expression of dehalogenase IVa of *Pseudomonas cepacia* MBA4 showed that the introduction of a His-Tag/thrombin cleavage site, N-terminal to the native protein, was able to mediate multimerization of the enzyme. A replacement of the thrombin cleavage site with an enterokinase cleavage site abolished this effect. Moreover, cleavage of the multimerized product by thrombin abolished the multimerization effect indicating the involvement of the thrombin cleavage site. Co-translation of the thrombin- and the enterokinase- cleavage-site-containing dehalogenase IVa produced active hybrid molecule migrating intermediate to the two parental molecules. This implied that the thrombin cleavage site could mediate a hetero-multimer formation for dehalogenases.

Detection of antibiotics by *Pseudomonas fluorescens* strain LRB3W1 and its role in antagonism and disease suppressiveness

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A strain of *Pseudomonas fluorescens* LRB3W1 isolated from lettuce roots had broad-spectrum antibiotic activity against various plant pathogenic fungi and bacteria, as well as growth promoting activity on tomato and asparagus. The strain produced several metabolites in culture including hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (Phl) (based on the analysis by UV, TLC, HPLC, NMR and GC-MASS spectra) and a fluorescent siderophore(FS).

LRB3W1 produced no detectable pyrrolnitrin, pyoluteorin or henazine-1-carboxylic acid (PCA). PCR analysis was conducted using primer pairs Phl2a/Phl2b and PCA2a/PCA3b which amplify specific sequences within the Phl and PCA biosynthetic loci, respectively. The Phl primers amplified a 745-bp fragment predictive of the Phl biosynthetic locus but the PCA primers amplified no fragment. Tn5 insertion mutants of LRB3W1 were screened for loss or decreased production of Phl and FS. Antibiotic activity of LRB3W1 and Tn5 mutants *in vitro* against *Rhizoctonia solani* (Rs), *Pythium* spp. (Pt), *Gaeumannomyces graminis* var. *tritici* (Ggt) and *Fusarium oxysporum* (FS) was as good as compared with the known antibiotics-producing *P. fluorescens* strains, though it varied with the indicators. Disease suppression by LRB3W1 was consistent against rhizoctonia root rot, caused by Rs AG-8, whereas it was inconsistent against take-all, caused by Ggt. Tn5 mutants, on the other hand, varied with the strains in both antibiotic activity and disease suppression ability.

No distinct correlation was existed between antibiotics productivity *in vitro* and disease suppression. Primers Phl2a/Phl2b amplified the predicted fragment from the DNA of Tn5 mutant of LRB3W1, although they were weak or negative in biochemical tests for Phl. PCR-RAPD analysis of LRB3W1 using a known random primer gave a different genomic banding pattern from other known *P. fluorescens* Phl-producing strains.

Elastolysis and rhamnolipid production in the absence of the transcriptional activator LasR

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In *Pseudomonas aeruginosa*, elastase and rhamnolipid production is controlled by at least two coordinate quorum sensing systems, the las and the rhl systems. Optimal elastase (*lasB*) and rhamnolipid (*rhlA*) expression requires the presence of the transcriptional activator LasR, and the Δ *lasR* mutant PAOR1 is known to be unable to produce elastase and rhamnolipid. We obtained an elastolytic mutant (PR1E4) of PAOR1 after two weeks of growth in a defined minimal media requiring elastase expression for growth. This mutant, in contrast to its parent strain, has a protease +, elastase +, and rhamnolipid + phenotype (measured by skim milk plates, elastin plates and SW blue agar plates respectively). Northern blot analysis of RNA obtained from PAOR1 and PR1E4, showed a marked increase of *lasB* and *rhlA* expression in PR1E4 compared to its parent strain. B-galactosidase assays using reporter fusions (*lasB-lacZ*, *rhlA-lacZ*) confirmed these results. Elastin congo red assays showed that elastase production in PR1E4 starts later (in early stationary phase), and reaches about 40% of the maximum elastase production of the wild type strain PAO1.

Our data supports the hypothesis that elastase and rhamnolipid production are possible in the absence of the transcriptional activator LasR.

Chromosomal Insertion of the *Ralstonia eutropha* JMP134 (pJP4) *tfdCDEF* Gene Cassette Allows Chlorocatechol Degradation in Aerobic Bacteria.

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Chlorosubstituted catechols are key intermediates produced by peripheral reactions of the metabolism of chloroaromatics. A plasmid encoded catabolic operon *tfdCDEF* from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134 (pJP4) allows this strain to degrade chlorocatechols (Don *et al.* J. Bacteriol. 161:85-90, 1985). In this work, two DNA cassettes containing the *tfdCDEF* operon under the control of promoter *P_{trc}* (inducible by IPTG) and *P_{sal}* (inducible by salicylate) were assembled in a mini-Tn5 delivery system (Herrero *et al.* J. Bacteriol. 6557-6567, 1990). This system allowed the stable chromosomal insertion of the DNA cassette into the pJP4-free derivative strain *R. eutropha* JMP222, a phenol degrader, and *Pseudomonas putida* KT2442, a benzoate degrader. In *Escherichia coli*, used as a control, the enzymes of the *lacI^q-P_{trc}-tfdCDEF* gene cassette were expressed semiconstitutively, and the enzymes of the *nahR-P_{sal}-tfdCDEF* gene cassette were inducible by salicylate, or 4-chlorosalicylate. In contrast, both gene cassettes were semiconstitutively expressed in strains *R. eutropha* JMP222, and *Pseudomonas putida* KT2442. The expression of the enzymes encoded by *tfdCDEF* gene allowed the use of 3-chlorobenzoate as sole carbon source in *R. eutropha* JMP222, but not in *P. putida* KT2442.

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187

MOLECULAR CHARACTERIZATION OF THE STYRENE UPPER PATHWAY FROM *Pseudomonas* sp. Y2

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Styrene, a colorless, volatile, strongly smelling compound which is toxic in fairly low quantities, is one of the most important aromatic chemicals produced industrially. Although styrene-degrading strains have been isolated several years ago, only very recently the enzymatic activities involved in this process have been identified and partially characterized. Up to now two main pathways have been shown for styrene degradation: one begins with the oxidation of the unsaturated side chain and the other involves the initial oxidation of the aromatic ring. However, only the genes responsible for the oxidation of the vinyl chain of styrene in *Pseudomonas fluorescens* ST have been cloned (1). Preliminary studies have shown that they are arranged into a cluster which involves a monooxygenase, an isomerase and a reductase that transform styrene into phenylacetic acid.

To increase the knowledge on the styrene catabolism we have investigated this process in *Pseudomonas* sp. Y2, a strain that has been reported to degrade styrene by a side chain oxidation mechanism (2). Taking advantage of the ability of the styrene monooxygenase to oxidize indol to the blue dye indigo, the genes encoding this enzyme have been cloned in *Escherichia coli* as a 13.5 kb-*EcoRI* fragment. A significant increase in the production of indigo was observed when the recombinant *E. coli* was grown in the presence of styrene. Analysis of the sequence of the 13.5 kb-*EcoRI* fragment and an overlapping 5.0 kb-*Clal* fragment, revealed the existence of four catabolic genes coding for a two component monooxygenase, an isomerase and a phenylacetaldehyde dehydrogenase, that will account for the oxidation of styrene to phenylacetic acid. Flanking the catabolic genes, a regulatory system and a potential membrane protein for the styrene uptake have been identified.

The upper pathway for styrene degradation reported here have been engineered as a DNA cassette that can be easily combined with that for the catabolism of phenylacetic acid, i.e. styrene lower pathway, and this will contribute to expand the catabolic abilities of different environmentally relevant bacteria to mineralize styrene.

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188

Genetic organization of sulfur-controlled aryl desulfonation in *Pseudomonas putida* S-313

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Sulfonated aromatic compounds (arylsulfonates) are industrially important compounds that are used as pharmaceuticals, detergents, dyestuffs and cement additives. A broad range of arylsulfonates can be desulfonated by *Pseudomonas putida* S-313, which was isolated from industrial sewage by enrichment with arylsulfonates as sole sulfur source (1). *In vivo* studies with this organism showed that desulfonation is catalysed by a monooxygenase system (1), and that expression of the corresponding genes is repressed during growth with sulfate, cysteine or thiocyanate (2). We have used the mini-Tn5 system to generate mutants of *P. putida* S-313 which are no longer able to utilize arylsulfonates as sulfur source. Five classes of mutants were obtained. Cloning and sequencing of the insertion region for Class I mutants revealed a cluster of three genes (*asfABC*), which from sequence homologies probably encode a multicomponent oxygenase involved in desulfonation, and includes the genes for a reductase, a ferredoxin, and a putative oxygenase. Upstream of *asfABC*, in divergent orientation, lies a fourth gene, *asfR*, which shows strong homology to regulator proteins of the LysR-type family.

Class V mutants were unable to grow with either aryl or alkyl sulfonates, and may therefore be defective in a regulatory locus. Genetic analysis revealed that the mini-Tn5 insertion had occurred in the *psuB* gene, in an operon with homology to *P. aeruginosa* and *E. coli* genes that are involved in alkanesulfonate metabolism. Class IV mutants cannot utilize either aromatic sulfate esters or arylsulfonates, and therefore constitute a second class of regulatory mutants, whereas Classes II and III show reduced or zero growth only for specific arylsulfonates, and probably represent transport mutants.

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189

Characterisation of a nitrogen-fixing *Pseudomonas stutzeri*

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In the eighties large screenings were set up to isolate N₂-fixing bacteria from the rhizosphere of paddy rice in China. One competitive, rice colonising diazotroph was characterised (mainly by nutritional and morphological features) as *Alcaligenes faecalis* A15 (You et al. 1991). When we started studies on the molecular aspects of nitrogen fixation and rice colonisation by this strain, we carried out a more in depth taxonomic analysis, based on 16S rDNA sequencing and fatty acid profiles. The A15 isolate is now reliably identified as *Pseudomonas stutzeri*.

Since the existence of nitrogen fixing pseudomonads still is not broadly accepted we decided to compare *P. stutzeri* A15 with other putative nitrogen-fixing *Pseudomonas* species, reported before: *Pseudomonas* sp. strain DC, isolated from *Deschampsia caespitosa* in Finland (Haahela et al. 1983) and *P. stutzeri* CMT.9.A, isolated from sorghum in Germany (Krotzky and Werner, 1987).

By analysing the restriction patterns of amplified ribosomal DNA (ARDRA-analysis) it was obvious that all the analysed strains are closely related with several *P. stutzeri* strains.

By PCR we amplified sequences specific for *Pseudomonas* spp. *strictu sensu* (*oprF* gene, Ullstrom et al. 1991), as well as *nifH* genes (the structural gene for the nitrogenase reductase). In a phylogenetic tree the partial *nifH* sequences of the diazotrophic *Pseudomonas* species are clustered and show homology with *Azotobacter nifH* genes, and also with *nifH* sequences generated by direct PCR of the rice rhizosphere (Ueda et al. 1995). The strong homology of the *oprF* sequences further substantiates the close taxonomic position of these strains, although isolated from completely different environments.

A few possible explanations for the ability of these *P. stutzeri* strains to fix nitrogen will be discussed: the strains might belong to a new, diazotrophic, genomovar of *P. stutzeri*, the *nif*-genes might be plasmid-borne, or, since *P. stutzeri* is known to be naturally competent, this might be a case of horizontal transfer of *nif*-genes by transformation and recombination.

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190

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TEXT NOT AVAILABLE

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As part of our analysis of the respiratory chain of *Pseudomonas aeruginosa* we have cloned and sequenced the genes required for production of a cytochrome c oxidase. The genes are partially homologous to the *ccoNOQP* and *ccoGHIS* sequences which encode, and are required for expression of a *cbh3*-type oxidase in *Rhodobacter* sp., *Paracoccus denitrificans* and *Rhizobiaceae*. In *P. aeruginosa* these genes lie upstream of *anr* and *hemN*, and the *ccoGHIS* gene cluster includes a fifth gene (*ccoI*) with no known homologue.

We will present the sequence analysis of these genes and the characterisation of mutants disrupted in *ccoI* (a putative copper-transporting P-type ATPase), *ccoS* and the novel *ccoI*. In particular we will discuss the effect these genes have on the activity of the cytochrome c oxidase.

A specific RFLP pattern of *hrp* genes of *Pseudomonas syringae* pv. *mori* detected by PCR-RFLP analysis in plant-pathogenic and epiphytic strains of *P. syringae*

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Plant pathogenic *Pseudomonas syringae* includes about 50 pathovars which are determined by their host range. RFLP patterns of *hrp* (hypersensitive reaction and pathogenicity) genes in 42 pathovars of *Pseudomonas syringae* were studied to detect pathovar-specific patterns. A part of *hrpD* (*Psp*) region of *P. syringae* pv. *phaseolicola* and a part of *hrpZ* (*Pss*) region of *P. syringae* pv. *syringae* were used as target regions to amplify by PCR. For PCR amplification of *hrpD* (*Psp*), D1(upstream)-D2(downstream) primer set reported by Scholz et al. (1) was used, and for *hrpZ* (*Pss*), another primer set was designed based on the sequence data (2). Twenty pathovars of *P. syringae* generated PCR products with predicted size (ca. 2.1 and 2.4kb) using the primer sets for *hrpD* (*Psp*) and also *hrpZ* (*Pss*), and 15 pathovars of *P. syringae* generated them only using the primer set for *hrpZ* (*Pss*). While, no product was amplified from other 7 pathovars of *P. syringae* and other pseudomonad by either primer sets. The PCR products thus obtained hybridized to pPL6 harboring *hrpA-S* region (3). These products were digested with two restriction enzymes, *MspI* and *HaeIII*, and analyzed. The PCR-RFLP analysis revealed that 42 pathovars were roughly classified into five groups and pv. *mori* strains were clearly distinguished from other pathovars. Then, 16 stock cultures isolated from diseased mulberry leaves including pv. *mori* and unidentified pathovars were examined for the RFLP pattern of *hrp* genes and the pathogenicity to mulberry, *Morus alba* cv. Kainezu. Five patterns of pv. *mori* were detected. All the strains exhibiting M1 and M1' (very similar to M1) patterns showed strong pathogenicity to mulberry plants. Other strains did not show the pathogenicity in this experiment. These patterns were different from any RFLP patterns of other pathovars strains. Furthermore, 27 strains of the epiphytic *P. syringae* isolated from healthy mulberry plants and 4 strains from the insect, mulberry pyralid, were investigated for their RFLP pattern of *hrp* genes. About 40% of these strains exhibited M1 and M1' patterns and strong pathogenicity to mulberry. Other strains exhibited several patterns which were different from any strains of *P. syringae* pathovars. These results revealed that the strains of pv. *mori* were distinguished from other pathovars by the RFLP pattern of *hrp* genes and that epiphytic *P. syringae* on mulberry leaves exhibited the variable patterns.

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193

A novel Gentisate 1,2-dioxygenase from *Sphingomonas* sp. strain RW5: Purification of the enzyme and cloning of its gene

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Gentisate (2,5-dihydroxybenzoate) is a key intermediate in the aerobic degradation of various aromatic compounds including dibenzofuranes, naphthalenes or salicylates and some of the chlorinated derivatives were assumed to be central intermediates in the degradation of some halogenated aromatics such as Dicamba. The microbial degradation of gentisate is initiated by the intradiol cleavage by gentisate 1,2-dioxygenase (gentisate: oxygen 1,2 oxidoreductase (deacyling)), forming maleylpyruvate which is then further converted to central metabolites of the Krebs cycle. Gentisate dioxygenases have been purified from crude extracts of different strains (Suarez et al., 1996; Suemori et al., 1993; Crawford et al., 1973; Harpel and Lipscomb, 1990). Until now there is no report on the localisation and characterisation of the gene encoding for the gentisate dioxygenase available.

We have now purified a gentisate 1,2-dioxygenase from crude extract of *Sphingomonas* sp. strain RW5 grown on Dicamba as sole carbon and energy source. The molecular mass of the purified holoenzyme was 194 kDa and its structure was deduced to be a tetramer with 43 kDa per subunit. By N-terminal and internal aminoacid sequencing of the enzyme we designed a pair of degenerate PCR primers. PCR was performed under low-stringency annealing conditions with total DNA from *Sphingomonas* sp. strain RW5 and resulted in a 1.2 kb PCR product. This product was used as a probe in a Southern blot of *Sphingomonas* sp. strain RW5 DNA cleaved with various restriction enzymes. A 4 kb DNA fragment was cloned showing a distinct activity for the conversion of gentisate. The resulting nucleotide sequence for a gentisic acid dioxygenase shows no close homology to other classI and classII dioxygenases, indicating its novel character.

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194

Intracellular growth of a *Pseudomonas aeruginosa* strain in polymorphonuclear leucocytes

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A mucoid *Pseudomonas aeruginosa* strain isolated from the airways of a patient with cystic fibrosis was found to express an unusual phenotype. These bacteria sustain 30 min incubation at 65°C and were found to be resistant to serum. In contrast to other CF- isolates this strain is susceptible to many phages which indicates a versatile cell surface, although no major differences were seen in the protein, phospholipid and fatty acid profiles of the membranes. The strain secretes large amounts of rhamnolipids and binds with high affinity and stereospecificity to the oligosaccharide moieties of mucins. Phagocytosis tests of this bacteria with polymorphonuclear leucocytes (PMN) yielded negative indices of phagocytosis and killing, regardless whether the PMN had been isolated from CF- patients or healthy donors. Electron microscopy unravelled the ability of intracellular growth within PMN. In contrast to other facultative intracellular bacteria, this *P. aeruginosa* strain was found to be able to divide in lysosomes without need for any time for adaptation. In some cases the phagosomal membrane became disintegrated and the bacteria grew in the cytosol. Furthermore cytosolic material was separated from the surroundings by bacterial membranes and subsequently the bacteria coated with host material were released from the leucocyte.

Four global regulators influence antibiotic production and stress response in *Pseudomonas fluorescens* Pf-5.

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Pseudomonas fluorescens Pf-5 is a rhizosphere bacterium that produces at least three antibiotics, including pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol, and suppresses seedling diseases caused by the soilborne fungi *Rhizoctonia solani* and *Pythium ultimum*. Three global regulatory genes are known to influence antibiotic production by Pf-5. *apdA* encodes a sensor kinase and *gacA* encodes a response regulator of a putative two component regulatory system; mutants with genetic lesions in either *apdA* or *gacA* produce none of the antibiotics. The stationary-phase sigma factor RpoS is a differential regulator of antibiotic biosynthesis genes; an RpoS⁻ mutant overproduces pyoluteorin and 2,4-diacetylphloroglucinol and produces no pyrrolnitrin. *ApdA*, *GacA*, and RpoS⁻ mutants were impaired in survival of oxidative stress compared to wildtype Pf-5. A study was initiated to determine the interactions of the three global regulators. At the entrance to stationary phase, *ApdA* and *GacA* positively influenced both the accumulation of RpoS, assessed by western analysis, and the induction of *rpoS* transcription, assessed with a transcriptional fusion to the chromosomal *rpoS* of Pf-5. In contrast, RpoS did not significantly influence accumulation of *ApdA*. These data suggest that *ApdA* and *GacA* regulate antibiotic production, and through their influence on RpoS, affect other phenotypes. Because *ApdA* and *GacA* mutants differ in the production of antibiotics from RpoS⁻ mutants, the two component regulatory system cannot act exclusively through RpoS. A fourth global regulator, a protease La homologue encoded by *lon*, negatively influenced pyoluteorin production but had no effect on the other two antibiotics produced by Pf-5. A *Lon* mutant was less capable than the wildtype Pf-5 of surviving UV damage; UV sensitivity is a characteristic phenotype of *Lon* mutants of *Escherichia coli*. We speculate that protease La represses pyoluteorin biosynthesis by degrading a positive regulator of pyoluteorin biosynthesis genes, such as the product of the recently identified *plrR* gene. Our results indicate that protease La, *ApdA*, *GacA*, and RpoS comprise four global regulators that influence both antibiotic production and stress response of *P. fluorescens*.

Lipolytic enzymes have recently attracted a great deal of attention because they have proven to be valuable tools for a variety of both hydrolytic and synthetic reactions of biotechnological importance. Interestingly, lipases originating from the genus *Pseudomonas* seem to be particularly suitable for biotechnological applications [1]. *Pseudomonas aeruginosa* secretes into the culture medium an extracellular lipase of M_r 29,000 which is encoded by the lipase gene *lipA* [2]. We observed that a lipase-negative deletion mutant still showed hydrolytic activity towards short chain length (C6) p-nitrophenylesters. A cosmid library of genomic DNA from *P. aeruginosa* PAO1 was conjugated into the mutant strain *P. aeruginosa* PABSI and transconjugants were screened for high esterolytic activity on tributyrin agar plates. The lipase-negative phenotype of the transconjugants was confirmed by using a lipase-specific plate assay, Southern blotting with a *lipA* probe, and immunoblotting with lipase specific antibodies. A lipase-negative clone showing high esterase activity was identified which contained a 3.3 kb *Xho*I fragment of chromosomal *P. aeruginosa* DNA. Analysis of the DNA sequence revealed an open reading frame of 1941 bp including a putative RpoN-dependent promoter located at position -135 and a ribosomal binding site at position -7 upstream the translational start codon ATG. This orf which was named *estA* and encodes a protein of 646 amino acids with a calculated M_r of 69,500 which has an N-terminal signal sequence of 24 amino acids. Expression of *estA* from plasmid pUCPSK in an *E. coli*-T7-system in the presence of [35 S]-methionine and subsequent analysis by SDS-PAGE and autoradiography revealed a protein band of M_r 66,000 corresponding to a mature EstA protein. This novel esterase belongs to a new family of lipolytic enzymes with unknown function which have been identified in a number of bacterial and plant species. A characteristic feature of these enzymes is the exchange of the classical lipase consensus motif GxSxG containing the active site serine residue by the N-terminally located motif GxSxxDxG [2]. Recently, we have succeeded to overexpress the *estA* gene in *P. aeruginosa* and we have started to purify and biochemically characterize this novel lipolytic enzyme.

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Soil bacteria capable of utilising 2-Bromopropionate (2MBPA) were isolated in basal salt minimal medium containing 2MBPA as the sole carbon and energy sources for growth. Soil samples were collected from various sites in Hong Kong and eight bacterial isolates were obtained. Only one strain was selected for further study. This bacterial strain was able to grow in minimal medium containing 2MBPA or 2-chloropropionic acid (2MCPA) as the only carbon and energy sources. The growth of the bacteria was accompanied by an increase in halide concentration in the culture media indicating the removal of halide from the halogenated substrates. However, this bacterial strain cannot grow in minimal media containing homologous halogenated alkanic acids of 2-carbons in length such as bromoacetic acid (MBA) and chloroacetic acid (MCA). This bacterial strain has been identified as *Pseudomonas putida* and the generation time, growing in minimal media using 2-bromopropionic acid, was 2.5 hour. Although the bacteria were not able to grow on MBA or MCA, cell free extract was found to be active toward all four 2-halo-alkanoic acids tested. Activity-stained polyacrylamide gel electrophoresis (PAGE) was used to detect the presence of dehalogenases, enzymes which are able to cleave the carbon-halogen bonding from dehalogenated substrates, in this bacterial isolates. Cell free extracts prepared from cultures grown on 2MBPA as the growth substrate were shown to contain a single dehalogenase. The mobility of this dehalogenase in the activity-stained PAGE was higher than that of Deh IVa (46 kDa) from *Pseudomonas cepacia* MBA4. This revealed that the native molecular weight of the dehalogenase was less than 46 kDa. 1 mm gel slices were cut from the native PAGE and subsequently analysed by SDS-PAGE. It was found that the denatured molecular weight of the dehalogenase was 30 kDa. Cell free extract prepared from *Pseudomonas putida* grown on lactate, succinate and pyruvate were also shown to produce dehalogenase. This indicated that the dehalogenase was produced constitutively.

Role of the outer membrane in *Pseudomonas aeruginosa* antibiotic susceptibility

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Pseudomonas aeruginosa has been shown to have very low outer membrane permeability coupled with secondary resistance mechanisms such as antibiotic efflux or an inducible chromosomal β -lactamase. Because of this low permeability it has been suggested that permeability of β -lactams does not occur via porin channels and does not follow the Zimmerman-Rosset equation. In contrast using an HPLC assay to measure outer membrane permeability of cephaloridine in cells overexpressing chromosomal β -lactamase, we could demonstrate a linear relationship between the rate of hydrolysis of cephaloridine in intact cells (rate limited by outer membrane permeation) and added concentrations of cephaloridine over a 60 fold range of concentrations (up to 10 fold the Km). This indicates that cephaloridine diffuses across the outer membrane.

It has been shown that a major co-contributor to antibiotic resistance in *P. aeruginosa* is the efflux operon *mexA mexB oprM*. This operon is basally expressed in wild type cells contributing to broad range intrinsic antibiotic resistance, and when overexpressed in *nalB* mutants leads to multiple antibiotic resistance. The antibiotics that serve as substrates for this pathway include quinolones, tetracycline, trimethoprim and chloramphenicol. However in *oprM* deficient mutants and *nalB* mutants β -lactam susceptibility is also affected, a surprising finding given that β -lactams have different characteristics to these above substrates and that the target site for β -lactams, the penicillin binding proteins, are proximal to and have a higher affinity than the cytoplasmic membrane into which the β -lactams are proposed to insert to join the *mexA mexB oprM* efflux pathway. To examine whether β -lactams served as a substrate for this pathway, or whether there was another explanation for these data, we first examined if net β -lactam uptake in wild type cells could be perturbed by energy inhibitors. KCN inhibition had no effect and we are currently assessing the affect of CCCP. Secondly, to see if *OprM* could be directly involved in β -lactam efflux from the periplasm, we overexpressed the cloned *oprM* gene in an *oprM::Tn501* background and obtained complementation of the *OprM* deficiency. However overexpressing *OprM* by itself in wild type cells had no effect on antibiotic susceptibility suggesting that *OprM* could not function independently in β -lactam efflux. Other studies looking at a potential role for β -lactamase will be discussed.

A genetic approach to isolate *AlgB*-dependent genes of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa strains involved in chronic respiratory infections in cystic fibrosis patients typically exhibit a mucoid colony morphology due to production of high levels of the exopolysaccharide alginate. Transcription of *algD*, the first gene in an operon encoding most of the alginate biosynthetic enzymes, is under complex transcriptional control. *AlgB*, which belongs to the NtrC/XylR family of response regulators, is required for high-level alginate synthesis and this effect has been linked to increased transcription from the *algD* promoter. Since repeated attempts to demonstrate binding of purified *AlgB* to the *algD* promoter region have been unsuccessful, it was hypothesized that *AlgB* exerts transcriptional control of *algD* indirectly either by activating transcription of a positive regulator or repressing expression of a negative effector. To address either of these possibilities, a nonmucoid *algB* mutant strain was mutagenized with Tn5-B50, a Tn5 derivative carrying an outward directed constitutive promoter. The rationale for this approach was that *AlgB*-activated positive regulators of *algD* transcription would be controlled by the Tn5-B50 promoter whereas *AlgB*-repressed negative effectors would be insertionally inactivated. Either situation should restore the mucoid phenotype to the *algB* mutant strain. Of 3,000 colonies harboring Tn5-B50 insertions, three mucoid derivatives were isolated. The locus containing the insertion of one was cloned, and the mutation was reconstructed in a clean *algB* mutant background resulting in restoration of the mucoid phenotype. This mutation was also constructed in the wild-type mucoid strain and in nonmucoid strains harboring mutations in either *algT* or *algR*, two other positive effectors of *algD* transcription, with no visible effect on the alginate phenotypes. This indicates the Tn5-B50 insertion affects alginate expression specifically in the *algB* mutant genetic background. The Tn5-B50 appears to have insertionally inactivated a novel *P. aeruginosa* gene designated *pepA*, encoding a protein with homology to bacterial proteases of the aminopeptidase family. By analogy with Lon proteolytic control of capsule synthesis in *E. coli*, a working hypothesis is that *AlgB* represses *pepA* but in *algB* mutants *pepA* is derepressed and the protease degrades an essential alginate regulator.

Strong expression of pore-forming cytotoxin is depending on the integration of phage ϕ CTX DNA into the genome of *Pseudomonas aeruginosa*

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Phage ϕ CTX, a member of the family *Myoviridae*, infects *Pseudomonas aeruginosa* (6 %) and *P. fluorescens* (5 %), but neither infects *Burkholderia cepacia* (N=40) nor *Staphylococcus aureus* (N=50). After infection the phage DNA was chromosomally integrated. The expression of *ctx*, which codes for the pore-forming cytotoxin, was at least a 100-fold higher in *P. aeruginosa* than in *P. fluorescens*. To analyse whether the difference in cytotoxin expression is depending on promoter activity, the ϕ CTX fragment *EcoRI*-B, containing *attP_{HA}*, *cos* and *ctx*, and deletion and point mutation variants were cloned into pHAI0. Recombinant plasmids carrying the chloramphenicol acetyltransferase gene were used. A σ^{70} -type promoter located upstream of *ctx* is responsible for the high expression of *ctx* in *P. aeruginosa* and can also be recognized by the transcription system of *E. coli*. The promoter activity of *P. fluorescens* is much weaker.

The average [G+C] content of the phage DNA fragments *EHI*5 (2.8 kb), *BV*1 (1.9 kb), *EcoRI*-D (2.6 kb), *EcoRI*-C (2.7 kb) and *B6* (2.3 kb) neighbouring the fragment *EcoRI*-B (3.3 kb) is 63.1 %, and therefore 9.3 % higher than the [G+C] content of *ctx*. This confirms the thesis of Hayashi et al. (1993)¹, indicating the *ctx* region as a reabsorbed part of ϕ CTX DNA.

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Phylogeny of rRNA group I *Pseudomonas* resolved by nucleotide sequence analysis of the *gyrB* gene

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Phylogenetic analysis of rRNA group I *Pseudomonas* (*Pseudomonas sensu stricto*) was conducted by using the nucleotide sequences of DNA gyrase beta subunit genes (*gyrB*). Partial nucleotide sequences of *gyrB* genes of about 100 *Pseudomonas* strains were determined by direct sequencing of PCR-amplified *gyrB* fragments. Phylogenetic trees based on *gyrB* sequences were reconstructed by using the neighbor-joining method and maximum parsimony method. rRNA group I *Pseudomonas* strains were resolved into the following four major subgroups:

- Subgroup 1: *P. aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. mendocina*, *P. oleovorans*, *P. straminea*
- Subgroup 2: *P. putida* biovar A, *P. fulva*
- Subgroup 3: *P. fluorescens* biovars (biovar I, II, III, and V), *P. putida* biovar B, *P. taetrolens*, *P. lundensis*, *P. chlororaphis* (*P. aureofaciens*), *P. aurantiaca*, *P. synxantha*, *P. azotoformans*, *P. mucidorens*, *P. tolaasii*
- Subgroup 4: *P. syringae* pathovars (pv. *syringae*, pv. *antirrhini*, pv. *coriandricola*, pv. *coronafaciens*, pv. *lachrymans*, pv. *morsprunorum*, pv. *phaseolicola*, pv. *pisi*), *P. viridiflava*

Genetic distances between each of these subgroups were less than 0.21 (*p*-distance: the proportion of nucleotide difference). Subgroup 3 contains diverse members and would be divided into several subordinate clusters. *P. fluorescens* biovars occupied various positions between the members of subgroup 3. Strains of *P. putida* biovar B were located within this *P. fluorescens* complex. Plant pathogenic *Pseudomonas* strains examined in this study were monophyletic (subgroup 4). *P. syringae* pathovars were divergent and formed a complex with *P. viridiflava*. The strains classified as *P. syringae* pv. *syringae* were scattered in the branch of subgroup 3 whereas races of *P. syringae* pv. *phaseolicola* were clustered. The type strain of *P. syringae* (Neopathotype strain of pv. *syringae*) formed a complex with the races of *P. syringae* pv. *pisi*. The results indicated the need to reclassify the rRNA group I *Pseudomonas*. Phylogenetic analysis using the nucleotide sequences of the *rpoD* genes (genes of sigma 70 factor) also supported this conclusion.

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Trichloroethylene (TCE) is a synthetic chlorinated solvent which was heavily used in the dry cleaning industry and is popular as a metal degreaser. This solvent is identified as the most-frequently-detected contaminant in groundwater and soil, and is the most-frequently-detected organic pollutant at Superfund-designated waste sites. A variety of recombinant bacteria have been constructed to provide a more useful regulatory system for the expression of TCE-degrading enzymes. These recombinant bacteria were shown to degrade TCE in the laboratory; however, *in situ* applications would most likely require the release of the recombinant bacteria into the environment. As a result, the TCE-degrading bacteria would be forced to compete against the native, wild-type bacteria already present in that environment. The ecological competitiveness of recombinant bacteria may be enhanced by utilizing the niche environment created by plant roots, or rhizosphere, which favors those microorganisms that have adapted to utilize the enzymes and nutrients exuded by the plant roots. In addition to improving the survival of the recombinant bacteria, the plant roots help to increase the availability of the pollutant by breaking and aerating soil particles as well as pumping TCE-containing water to the root-colonizing bacteria. Even more importantly, the roots help supply oxygen, the limiting substrate required for TCE mineralization by bacterial oxygenases. In turn, these microorganisms can benefit the plant by decomposing dead plant matter, decreasing the vulnerability of the roots to pathogens, and synthesizing growth factors. This bacteria-plant interaction has been utilized in this study to provide a competitive advantage to root-colonizing bacteria genetically-engineered to degrade TCE as well as to create a low-cost, low-maintenance *in situ* TCE remediation method.

To prove this concept, a wheat-colonizing bacterium (*Pseudomonas fluorescens* 2-79) has been engineered to constitutively express the TCE-degrading enzyme, toluene *ortho*-monooxygenase (TOM), by integration of *tomA* into the chromosome of the root-colonizing pseudomonad. The resultant recombinant strain stably degrades TCE for over 280 generations without antibiotic selection pressure, while expressing active TOM (1 nmol TCE/min-mg protein) at levels comparable to the original strain (*Burkholderia cepacia* G4 PRI) from which *tomA* and its constitutive promoter were isolated. The recombinant root-colonizing bacteria are capable of colonizing wheat roots at densities (~3x10⁶ cfu/cm root) similar to the wild-type *P. fluorescens* 2-79 and are robust since they grow at the same rate as the wild-type strain. Additional data will be presented showing the competitiveness of this genetically-engineered strain in the rhizosphere against non-root-colonizing, soil bacteria. The effectiveness of the bacterial/soil/plant interaction to degrade 55% of the initial TCE has been demonstrated using microcosms in which the recombinant bacterium (introduced into the soil by coating wheat seeds with the pseudomonad prior to planting) is maintained in the rhizosphere of the cultivated wheat. These microcosms were compared to negative controls that showed a maximum 10% loss of initial TCE after seven days (including sterile soil only, soil with uninoculated wheat, and soil with wheat inoculated with the wild-type *P. fluorescens* 2-79). TCE degradation data from microcosms of soil initially inoculated with the recombinant bacteria before wheat planting will also be shown.

203

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The processing of crude oil generates a number of leftovers among which the so called "heavy aromatic residues" stand out because of their toxicity. These residues are a complex mixture of high molecular weight hydrocarbons, and contain a high percentage of polyaromatic compounds (around 20%) and substantial amounts of vanadium. Given their toxicity and that many of the polyaromatic compounds present are potential carcinogens, their disposal poses an environmental problem. For this reason they are normally incinerated. To search for methods to either degrade, detoxify or even exploit these kind of residues we have isolated and characterized a number of bacterial strains able to use them as a carbon source. We obtained both Gram negative and Gram positive isolates that could thrive on the residue with generation times ranging from 4 hours to 60 hours. Among the Gram negative isolates, strains of *Pseudomonas aeruginosa*, *Burkholderia* (*Pseudomonas*) *cepacia* and a putative *Pseudomonas vesicularis* were found. Most of the strains either emulsified or changed the viscosity of the oil residue. None of them could grow at the expense of a number of purified polyaromatic compounds that were found to be present in the residues, although all seemed to modify the corresponding compound when growing on the oil residue. This suggests that cells may be cometabolizing these polyaromatics. The oil residue contains large amounts of high molecular weight alkanes and alkyl-aromatic compounds which we believe are used by the strains as carbon sources when growing at the expense of the residues. Indeed, all the strains could use as sole source of carbon and energy a wide range of purified high molecular weight alkanes and alkyl-aromatic compounds, and some of them could also degrade branched alkanes. This opens up possible applications of the strains for the treatment of the residues. We are currently studying the pathways for alkane degradation of the isolates characterized as *Burkholderia cepacia* and *Pseudomonas aeruginosa*.

204

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Over the past 30 years, a number of *Pseudomonas* spp. have been isolated on alpha pinene as a sole carbon source. These have been shown to accumulate some potentially valuable intermediates, such as carvone and (+) borneol during growth on terpenes, but usually these are minor components of a complex mixture of products and may be side products of the main catabolic pathways.

As part of an EEC programme, we are investigating the possibility of creating strains by a combination of mutagenesis, gene cloning and reverse genetics, which accumulate specific single metabolites in high yields. Two programmes of work will be described in this poster:
(i) Heterologous expression of alpha pinene monooxygenase and epoxide lyase from *Pseudomonas fluorescens* NCIMB 11671. (ii) Isolation and characterisation of mutants of *Pseudomonas* sp. PL (NCIMB 12693) including analysis of metabolite accumulation.

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Of ten strains of *B. pseudomallei* tested only one, strain 576¹, became electro-competent using standard preparative methods as described for *Escherichia coli*². All others tested needed the addition of low levels of the aminoglycoside antibiotic kanamycin to their growth media before preparation³. Strain 576 was transformed using the plasmid pKT230 (Kan^r) which remained stable in this host. The transformation frequency was calculated using pKT230 and compared to that obtained with the other strains which had been prepared using kanamycin in their growth medias. The frequency of transformation was found to be two logs higher. A suicide plasmid carrying the transposon mini Tn5 was used to transform strain 576 by electroporation. Integrants were isolated and Southern Blots performed to demonstrate the random nature of the insertion of this transposon into the *B. pseudomallei* genome.

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The use of antibodies to characterise the changes in *Pseudomonas* population during spoilage of milk

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Storage of milk products at refrigeration temperatures favours the growth of psychrotrophic bacteria which produce proteolytic and lipolytic enzymes and hence can cause quality problems in the dairy industry. Psychrotrophic micro-organisms are capable of growing at 1°C and are represented by both Gram positive and Gram negative bacteria. The Gram negative *Pseudomonas* spp. usually represents 10% of the microflora of freshly drawn milk, but at the end of product shelf-life they are the most numerous members of the psychrotrophic population.

Antibodies were raised against *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, isolated from raw milk and chicken respectively, characterised and used to follow the changes in a *Pseudomonas* sub population of either naturally occurring *P. fluorescens* or introduced *P. aeruginosa* during the spoilage of milk products including semi-skimmed milk, full fat milk and cream.

For *P. fluorescens* the antibody staining population correlated well with total *Pseudomonas* count as enumerated throughout the spoilage period on *Pseudomonas* CFC agar, indicating that this is the major species causing spoilage at 4°C. With the introduced *P. aeruginosa*, *P. aeruginosa* antibody cross-reacting colonies dominated for the first 2 days but their dominance in the flora decreased between day 3 & 7 then re-established again as the dominant strain on day 8. This demonstrates that bacteria introduced into foods do not grow in the same manner as the existing flora until a period of adaptation has taken place indicating that studies using introduced micro-organisms should be treated with caution.

207

Engineering of chlorotoluene degrading microorganisms.

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The 1,2,4-trichlorobenzene degrading strain *Burkholderia* sp. PS12 (1) was reported to mineralize chlorosubstituted benzenes and also 4-chloro- and some dichlorotoluenes. 2- and 3-chlorotoluene were not growth substrates. In contrast to 1,2,4-trichlorobenzene and 4-chlorotoluene, which are subject to dioxygenation by tetrachlorobenzene dioxygenase of *Burkholderia* sp. PS12, this enzyme acts as a monooxygenase on 2- and 3-chlorotoluene. The formed benzylalcohols are converted only very slowly to the carbonic acids. As the resulting 3-chlorobenzoate can be used as growth substrate, the failure to grow with 3-chlorotoluene can be assumed to be due to the low transformation rate of the alcohol/aldehyde. In the case of 2-chlorotoluene missing activity with 2-chlorobenzoate is a major pathway bottleneck. Consequently for the degradation of chlorotoluenes four different degradation-sequences are required: a chlorobenzene dioxygenase, two active dehydrogenases to transform chlorobenzylalcohols into chlorobenzoates, a 2-chlorobenzoate dioxygenase to form the catechol and enzymes for the mineralization of chlorocatechols. *Burkholderia* sp. PS12, harbouring chlorobenzene dioxygenase and a chlorocatechol pathway, and *Pseudomonas putida* F1 harbouring a toluene dioxygenase, were chosen as recipients for complementation studies using the following pathway segments: a gene cassette containing genes of the TOL upper pathway, a gene cassette (2) based on the genes coding for chlorocatechol mineralizing enzymes from *Pseudomonas* sp. pP51 (3) and a gene cassette encoding 2-chlorobenzoate dioxygenase derived from *Pseudomonas cepacia* 2CBS (4). Growth characteristics of natural and constructed microorganisms, as well as activity of pathway modules under different growth conditions and existing pathway bottlenecks will be reported.

References:

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208

Field testing of biologically contained *Pseudomonas putida* strain with biodegradative potential.

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Active biological containment (ABC) systems have been conceived as a way to control the survival of microorganisms under environmental conditions. Such systems are made of two elements: A killing element designed to induce cell death, and a control element which modulates the expression of the killing function. *Pseudomonas putida* CMC4 is a biologically contained strain that carries the elements of an ABC system on the host chromosome. This strain was designed so that it survived in the presence of 3-methylbenzoate and committed suicide in the absence of the aromatic. The behavior of the strain was confirmed in the laboratory and in field tests in two releases that covered the spring-summer and the autumn-winter periods. In the releases we studied the survival of the contained strain and an uncontained control strain in planted and unplanted soil with and without 3-methylbenzoate. In nonvegetated soils both contained and noncontained strains tended to disappear, but the contained strain disappeared faster in soils without 3-methylbenzoate. In the spring-summer period disappearance was faster than in the autumn-winter time. In vegetated soils survival was studied in the rhizosphere and in bulk soil. In the rhizosphere of plants the uncontained strain tended to establish at levels on the order of 10^6 CFU/g soil regardless of the presence of 3-methylbenzoate. In the bulk soil numbers were 2 to 3 orders of magnitude lower. In vegetated soils the contained strain tended to disappear, but this was more pronounced in the absence of 3-methylbenzoate, and was faster in the summer assay than in the winter one. We found no evidence of dispersal outside the experimental plots.

209

CymR, a transcriptional repressor of the cymene-cumate operon in *Pseudomonas putida* F1

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Pseudomonas putida F1, better known for its dioxygenase pathway for the degradation of toluene (*tod*), also encodes a degradative pathway for *p*-isopropyltoluene (*p*-cymene). This companion pathway consists of nineteen genes organized in an operon which we have designated *cym*. We have identified a major and internal promoter (*P_{cym}* and *P_{cum}*, respectively) for the *cym* operon that is induced by cymene and its benzoate derivative, *p*-cumate.

We show that a 28 kDa protein, termed CymR and encoded by an open reading frame upstream of the *cym* operon, functions as a repressor that inhibits the expression of these promoters under non-inducing conditions.

Inactivation of the *cymR* gene by kanamycin gene insertion results in constitutive expression as judged by the growth characteristics of the strains and enzyme activity assay. The repressor was overproduced in *Escherichia coli* as a GST fusion protein and purified by the glutathione-affinity column. The purified protein was found to bind specifically to DNA fragments containing the two promoters. DNaseI footprinting experiment revealed that CymR protects a 20-bp DNA region having an imperfect inverted repeat sequence which begins at about 10 bases downstream of the -10 regions of the two promoters. Gel retardation assays showed that binding of CymR to the promoters was inhibited in the presence of cumate. It is concluded that CymR represses transcription by binding to the operator site in the absence of the effectors such that effective binding of RNA polymerase is prevented. CymR is a new member of the MirR group of repressor proteins which in general control the level of bacterial cellular susceptibility to toxic hydrophobic substances in the environment.

210

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Several exported enzymes, are maximally produced in *P. fluorescens* when the growth temperature is lower than the optimum growth temperature, and averages 17°C. Evidences had been provided that, at least in some cases, this is due to a transcriptional regulation. By using insertion -fusion transposon mini Tn5lacZ1, we have obtained genes that display this special kind of temperature regulation, some of which are involved in the production of the extracellular enzymes, such as lipase, and protease.

Using the same strategy, we have found a partial activator of two of these genes, which is itself maximally expressed at low temperature (8°C). The protein sequence deduced from part of this gene is homologous to that of the large exonuclease VII sub-unit from *E.coli* or *B.subtilis*. On the other hand we have cloned a gene (*est*) that encodes the extracellular esterase from *P. fluorescens*, and the corresponding upstream sequences. Reverse genetics allowed us to insert a 'lacZ' transcription fusion into this gene which will permit to study its temperature regulation, and the role of the upstream sequences in this regulation.

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The gene encoding the peptidoglycan-associated outer membrane lipoprotein (PAL) of *Pseudomonas putida* has been cloned and sequenced (1). This gene, called *oprL* in *Pseudomonas*, was shown to be conserved in a wide variety of the *Pseudomonas* strains belonging to the rRNA group I. *OprL* null mutants of *P. putida* strain KT2440 and of the toluene-tolerant strain *P. putida* DOT-T1 were generated by reverse genetics and their phenotype were analyzed. When compared with the parental strain, the *oprL* mutant of *P. putida* KT2440 was more sensitive to sodium dodecyl sulfate, deoxycholate and EDTA, and exhibited an altered cell envelope, as revealed by electron microscopy (2). The *oprL* mutant of *P. putida* DOT-T1 became hypersensitive to toluene, accumulating from 25- to 50-fold more aromatics in its membranes than the parental strain (3). These results suggest that the *oprL* gene is important in maintaining the integrity of the cell envelope.

The region around the *oprL* gene has been sequenced. Upstream of *oprL*, five open reading frames have been identified that show homology with the *orfI*, *tolQ*, *tolR*, *tolA*, and *tolB* genes (which are also found upstream of *pal* in *Escherichia coli* and *Haemophilus influenzae*), and with the *tolQ*, *tolR*, and *tolA* genes of *P. aeruginosa*. Downstream of *oprL*, another open reading frame homologous to the *orf2* gene of *E. coli* and named *orf2* was found.

We are currently generating mutants in these genes to study their function in *P. putida*. The ability of the *P. putida tol* genes to complement *E. coli tol* mutants is being tested in order to compare the Tol systems of the two bacteria. We will present data on the transcriptional organization of the *orfI-tolQRAB-oprL-orf2* gene cluster of *P. putida*.

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Biodegradation of nitriles and 2,4,6-trinitrotoluene (TNT): two main contaminants in industrial effluents

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Among synthetic nitroorganic chemicals, nitriles and TNT have been declared by several environmental protection agencies as pollutants whose elimination is a priority and Governmental bodies. Nitriles are extensively used as solvents in the manufacture of plastics, synthetic fibers, dyestuffs, pharmaceuticals and herbicides, and TNT is the most widely used civil and military explosive. Removal of these chemicals from industrial effluents and soils is of the highest interest. *Pseudomonas* sp. VI-1 is a strain able to use wastewater from an acrylic fiber factory as the sole source of carbon, nitrogen and sulfur. The removal of compounds in the effluents by *Pseudomonas* sp. clone VI-1 was analyzed by HPLC and found to be complete for some (but not all) chemicals. A decrease of more than 60% chemical oxygen demand (COD) was achieved. Nitrile metabolism was studied with propionitrile as a model compound. This compound was mineralized by *Pseudomonas* sp. VI-1 via propionamide and propionic acid. *Pseudomonas* sp. JLR11 was isolated from a wastewater treatment plant as able to use TNT as the sole nitrogen source under anaerobiosis. Analyses of nitrogen balances showed that more than 80% of the nitrogen from TNT was incorporated into the cell biomass. Removal of N from the TNT ring did not involve progressive reduction of TNT to 2,4,6-triaminotoluene, but rather most likely involved the removal of nitrite. Some of the N-free C-rings from TNT were also mineralized, and hydroxylated metabolites were found as intermediates.

Expanding the catabolic range of *Pseudomonas* sp. PS12 by the recruitment of the *bed* gene cluster from *P. putida* ML2

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Two *Pseudomonas* species, *Pseudomonas* sp. PS12 and *P. putida* ML2 are able to degrade chlorobenzenes and benzene, respectively. Using a Tn5 mini-transposon the genes encoding benzene dioxygenase (*bedC1C2B4*) and *cis*-benzene dihydrodiol dehydrogenase (*bedD*), the first two enzymes involved in benzene degradation in *P. putida* ML2, were introduced into *Pseudomonas* sp. PS12. Five recombinant strains were obtained, each with an added ability to utilize benzene as sole carbon and energy source.

Southern analysis of the genomes of each recombinant strain confirmed the presence of the *bed* gene cluster. Using GC-MS analysis the intermediates of benzene metabolism, namely *cis*-benzene dihydrodiol and catechol were detected indicating the functionality of the cloned genes in the recombinant bacteria. An interesting observation was the detection of a ring-hydroxylating dioxygenase in *Pseudomonas* sp. PS12 which is able to recognize benzene as substrate, the genes of which bear homology to *bedC1C2B4* from *P. putida* ML2.

The stability of the cloned genes was tested under non-selective and substrate-limiting conditions in a chemostat. The results showed that the *bed* gene cluster was stably maintained in the recombinant strain for at least 15 generations.

**ALPHABETICAL
INDEX OF
CONTRIBUTORS**

NAME

ABSTRACT Number

NAME

ABSTRACT Number

| | | | | | |
|--------------|----------|-------------------------|-------------|-------|----------|
| Aarons | S. | XXIII | Balashova | N. V. | 98 |
| Abdelal | A. T. | 127, 139 | Ball | G. | 13 |
| Abe | M. | XXX | Bally | M. | 13, 28 |
| Abeyasinghe | P. | XXIV | Balzer | A. | 201 |
| Adewoye | L. | 1 | Bamford | D. | 34 |
| Ahey-Pollard | A. | 8 | Bantinaki | E. | 14 |
| Aizpun | M. | 167, 168 | Baquero | F. | XXIX |
| Akahiru | S. | 7, 123 | Barbieri | P. | XII, 9 |
| Akutsu | Y. | 2 | Barnes | M. R. | XV |
| Alaminos | M. | 62 | Bartels | F. | 15 |
| Allaria | M. | 49 | Bazhanov | D. | 16 |
| Allen | D. | 34 | Bazhanova | A. A. | 16 |
| Allen | C. C. R. | 90 | Beltrametti | P. | 41 |
| Alm | R. A. | XXI | Bénédetti | H. | 212 |
| Alonso | S. | 188 | Bennasar | A. | 68 |
| Alonso | A. M. | 3, 4 | Berlanda | D. | 9 |
| Altmann | A. | 108 | Bertoni | G. | XIX, XII |
| Andersen | S. M. | 5 | Bestetti | G. | 41 |
| Andersen | J. B. | VIII, 29 | Bevino | A. | 33, 178 |
| Anjaiah | V. | 6 | Beyeler | M. | 17 |
| Arai | H. | 7, 76 | Bignell | C. | 18 |
| Arenghi | F. | XII, 8 | Birmingham | A. | 23 |
| Armengaud | J. | 9, 81 | Bitter | W. | 99 |
| Arnold | D. L. | 10, 60, 87, 202 | Bleves | S. | 13 |
| Ashelford | K. | 11 | Bloemberg | G. | XX |
| Atkins | T. | 206 | Blumer | C. | 19 |
| Attanasio | T. | 69 | Boldt | T. S. | 20 |
| Averhoff | B. | 89 | Bolognese | F. | XII |
| Bacaicoa | B. | 167 | Bombaci | M. | 70 |
| Baele | M. | 24 | Booth | Z. | 207 |
| Bagneris | C. | 12 | Boronin | A. M. | 52, 98 |
| Bailey | M. | 11, 14, 44, 45, 61, 181 | Borremans | B. | XI |
| Bailey | A. | 142 | Bosch | R. | 21 |
| Bakiene | E. | 35 | Bouwman | C. W. | 22 |
| Bakker | P. | 117 | Braun | E. | 82 |

| | | | | | |
|--------------|-------|----------------------|------------------|---------|-----------------------|
| Brazil | G. M. | 23 | Covert-Rinaldi | A. | 71 |
| Brenner | V. | 140, 149, 182 | Culbret | E | 9 |
| Brown | N. | XI | Curty | L. K. | 97 |
| Buemi | C. | 69 | D'Argenio | D. | XIII |
| Bürger | C. | XXVIII | D'auria | G. | 70 |
| Burini | J. F. | 211 | Dalmastri | C. | 33, 178 |
| Buysens | S. | 24 | Darrah | P. | 61 |
| Byrne | A. | 133 | Darzens | A. | 34 |
| Cabrera | J. E. | 25 | Daugelavicius | R. | 35 |
| Cahan | R. | 26 | Day | M. J. | 11, 14, 45 |
| Campanario | E. | 4 | Dé | E | XLII, 54 |
| Campbell | J. | 116 | De Bont | J. A. M | XXXVIII, 84 |
| Canal | A. | 168 | De Cock | H. | 99 |
| Canduela | J. | 150 | De Kievit | T. | XXVII, 36 |
| Carazza | S. | 70 | De Lorenzo | V. | XVI, XIX, 15, 59, 106 |
| Caro | A. D. | 172 | De Mot | R | XLII, 32, 190 |
| Cases | I. | XVI | De Vicente | A. | 27 |
| Castillo | T. | 191 | De Vos | D. | 37 |
| Cazorla | F. M. | 27 | De Weger | L | XX |
| Céspedes | R. | 187 | Dehò | G. | 49 |
| Chakrabarty | A. M. | XXXI, 132 | Dekkers | L | XX, 38 |
| Chaney | N. | 130 | Delany | I | XXIII |
| Chapon-Herve | V. | 13, 28 | Delic-Attree | I | 183 |
| Chiale | C. | 50 | Dennerova | K | 149, 182 |
| Chiaroni | L | 33, 178 | Denning | N. F. | 39 |
| Christiansen | B. | VIII, 29 | Dennis | J. | 40 |
| Cisterna | R. | 150 | Denny | T. | XXII |
| Clough | S. | XXII | Deretic | V. | III |
| Coifu | O. | 116 | Demedde | J. | 121 |
| Colombo | C. | 41 | Devingilis | C. | 85 |
| Corbell | N. | 30 | Di Gennaro | P. | 41 |
| Corbella | M. E. | 31 | Di Ilio | C | 48 |
| Corbister | P. | XI | Di Le Cce | C. | XII |
| Corkery | D. | 32 | Di Paola | R. | 69 |
| Cornaglia | E | 115 | Díaz | E | 59, 188 |
| Cornelis | P. | XXIV, 6, 37, 57, 120 | Díaz de Villegas | M. E. | 191 |
| Costerton | J. W. | X | Ding | Q. | 37 |

| | | | | | |
|--------------|--------------|------------------|------------------|-----------|-------------------|
| Ditty | J. L. | XXXX | Frelet | M. A. | 54 |
| Dobson | A. | 65, 134 | Fry | J. C. | 11, 45 |
| Dodd | C. | 207 | Fuenmayor | S. | 55 |
| Domingo | A. | 63, 64 | Fukunori | F. | 56 |
| Dougan | G. | 206 | Furukawa | K. | XXXIV, 125 |
| Dowling | D. | 23 | Gaballa | A. | XXIV, 57, 120 |
| Drew | R. | XXXV, 129 | Gal | M. | 58 |
| Duetz | W. | XV | Gallardo | M. E. | 59 |
| Duque | E. | 62 | Gallego | L. | 150 |
| Dyke | K. | 44, 181 | Gallegos | M. T. | XVII |
| Eaton | R. | 42 | Galli | E. | XII, 9, 41 |
| Eberl | L. | 29 | Gamalero | E. | 115 |
| Ekpo | P. | 43, 145 | García | L. | 191 |
| El Hamel | C. | 54 | García | R. | 191 |
| Ellis | R. | 44, 181 | García | J. L. | 59, 188 |
| Eltis | L. D. | XXXII | García-Valdés | E. | 21 |
| Esteve-Nuñez | A. | 213 | Gardiner | S. | 23 |
| Evans | A. S. E. | 45 | Garrido-Perterra | M. E. | 31 |
| Evans | K. | XLIV | Garufi | G. | 70 |
| Farmer | S. | 199 | Gedney | R. M. | 42 |
| Farmer | K. | 46, 47 | Genovese. | T. | 68 |
| Favaloro | B. | 48 | Genti-Raimondi | S. | 25 |
| Favaro | R. | 49 | Gibbon | M. J. | 8, 87 |
| Fenton | A. | XXIII | Gibson | D. T. | XXXVI |
| Fernandez | A. | 59 | Gilmartin | C. | 60 |
| Fernández | M. C. | 50 | Givskov | M. | 116 |
| Ferri | A. | 69 | Gloux | K. | 163 |
| Ferns Pérez | M. A. | 51 | Goddard | V. | 61 |
| Filloux | A. | 13 | Godoy | P. | 62 |
| Fillonov | A. | 52 | Goh | G. | 53 |
| Fischer | P. | 156 | Goldberg | J. | VI, 172 |
| Flavier | A. | XXII | Gómez | D. E. | 63, 64 |
| Fleury | D. | 163 | González | B. | 187 |
| Fogliano | M. | 28 | Gordon | L. | 65 |
| Fong | P. Y. | 53 | Gorenflo | V. | XL |
| Forest | E. | 54 | Gotoh | N. | 66 |
| Frade | H. | 50 | Gottschalk | G. | 89 |

| | | | | | |
|------------------|----------|--------------------|------------|-------|---------------------|
| Greated | A. | 67 | Holby | N. | XXVI, 116 |
| Grimm | A. C. | XXXIX | Holte | A. | 15 |
| Grolms | U. | 158 | Homma | Y. | 185 |
| Guasp | C. | 68 | Hōrak | R. | 93, 180 |
| Guespin-Michel | J. F. | 211 | Horikoshi | K. | 56 |
| Gügi | B. | 211 | Horita | M. | 79, 185 |
| Guglielmino | S. | 69, 70 | Hozumi | T. | 128 |
| Haas | D. | 18, 19 | Huang | H. | XLI |
| Hager | P. | 71, 72, 148 | Huang | J. | XXII |
| Hales | B. A. | 73 | Huertas | M. J. | 62 |
| Hammer | P. | 108 | Huët | J. | 80 |
| Han | B. | 74, 171 | Hugo | N. | 81 |
| Han | E. M. | 75 | Hulen | C. | 80 |
| Hancock | R. E. W. | XLI, 131, 199 | Hungerer | C. | 82, 157 |
| Happe | B. | 88 | Ibañez | S. | 50 |
| Harayama | S. | XXXIII, 83, 202 | Igarashi | Y. | 76 |
| Hart | C. A. | 39, 73 | Iglewski | B. | XXVII, 36, 141, 186 |
| Harwood | C. S. | XXXIX | Ioue | J. | 83 |
| Hayashi | N. | 76 | Isken | S. | 84 |
| Hein | S. | XL | Itai | A. | 83 |
| Heinaru | A. | 143 | Itoh | Y. | 127 |
| Heinaru | E. | 143 | Iurescia | S. | 85, 111 |
| Heipieper | H. J. | 85 | Iwasaki | K. | 86 |
| Heiss | G. | 156 | Jackson | B. | 205 |
| Hernandez | J. | 51 | Jackson | D. A. | 65 |
| Hernández | M. | 51 | Jackson | R. W. | 87, 202 |
| Herrera-Estrella | L. | 142 | Jacob | S. | 103 |
| Herrmann | H. | 104, 121, 122, 195 | Jacobsen | C. S. | 5, 20 |
| Hester | K. | 77 | Jaeger | K-E | 105, 197 |
| Hettwer | U. | 78, 158 | Jahn | M. | 119 |
| Heuer | T. | XXVIII | Jahn | D. | 119, 157 |
| Hill | S. | 108 | Jakobs | B. | 88, 96 |
| Hoang | T. T. | XLIII | James | K. | XIV |
| Hobbs | M. | XXI | Jansen | D. B. | IX, 112 |
| Hobman | J. | XI | Jansen | S. | XXVIII |
| Höfte | M. | 24 | Jaquinot | M. | 54 |
| Hohaus | K. | 108 | Jendrossek | D. | 166 |

| | | | | | |
|-----------|-------|------------------|---------------|-------|------------------|
| Jensen | P. | 116 | Kosheleva | I | 98 |
| Johann | A. | 89 | Koster | M. | 99 |
| Johnson | G. | 133 | Kraak | M. | 100 |
| Johnson | S. | 207 | Kravchenko | L. | XX |
| Johnson | M. | 90 | Kresse | A. | 137 |
| Johnston | K. | 74 | Kubesch | P. | 195 |
| Johnstone | K. | 171 | Kudo | T. | 7 |
| Jorgensen | C. | 5 | Kukor | J. | 133 |
| Jouanneau | Y. | 81 | Kulakov | L. | 90 |
| Jung | Y. H | 75, 91, 147 | Kumamaru | T. | XXXIV |
| Kahn | S. | 92 | La Porta | R. | 69 |
| Kaldalu | N. | XVIII | Lalucat | J. | 68 |
| Kallastu | A. | 93 | Lamovskaya | O. | XLIV |
| Karlson | U. | 126 | Langenbach | S. | 101 |
| Karpov | A. V. | 52 | Langewische | F. | 201 |
| Kasak | L. | 93 | Langsrud | S. | 102 |
| Kasberg | T. | 112 | Larkin | M. J. | 90 |
| Kaschabek | S. R. | IX, 112 | Latifi | A. | 28 |
| Kawasumu | T. | 86 | Latijnhouwers | M. | 99 |
| Keel | C. | 18 | Lau | P. | 210 |
| Kembla | M. | 96 | Patel | A. M. | 210 |
| Kertes | M. | 189 | Wang | Y. | 210 |
| Kessler | B. | 100, 147, 155 | Hasegawa | Y. | 210 |
| Kessler | E. | 26, 94 | Laue | B. | 103 |
| Kharazmi | A. | 116 | Lauf | U. | 104 |
| Kikuchi | M. | XXXIII | Laux | P. | 158 |
| Kimura | N. | XXXIV | Laverack | P. D. | 103 |
| Kindler | B. | 173 | Laville | J. | 19 |
| Kirner | S. | 108 | Lazdunski | A. | XXV, 13, 28, 212 |
| Kivisaar | M. | 93, 180 | Le Rouzic | J. | 163 |
| Kiyono | M. | 95 | Leak | D. | 205 |
| Klem | T. J. | 128 | Lee | M. S. | 75 |
| Kodama | T. | 76 | Lehning | A. | 208 |
| Koedam | N. | XXIV, 6, 57, 120 | Leipelt | M. | 105 |
| Kohler | T. | 97 | Leisinger | T. | 189 |
| Kon | T. | 175 | Leoni | L. | 106 |
| Koopmann | B. | 78 | Li | X. | XLIV |

| | | | | | |
|--------------------|----------|-----------------|----------------|----------|-------------------------|
| Liehe | A. | 107, 158 | Mc Askie | L. E. | 67 |
| Ligon | J. | 108 | Mc Cusker | M. P. | 131 |
| Lilley | A. | 11, 14 | Mc Fall | S. | 132 |
| Lingner | M. | 195 | Mc Farlane | E. | XLI |
| Lloyd-Jones | G. | 170 | Mc Garvey | J. | XXII |
| Loh | H. L. | 214 | Mc Iver | K. | 94 |
| Loper | J. E. | 6, 30, 130, 196 | Mc Pherson | C. | IV |
| López | F. | 150 | Mercado Blanco | J. | 117 |
| Lorenzo | M. | 191 | Meragey | M. | XI |
| Lu | C-D | 127, 139 | Mereau | A. | 211 |
| Luciano | L. | 195 | Meyer | A. | 118 |
| Lugtenberg | B. J. J. | XX, XLII, 38 | Meyer | J. M. | 120, 176 |
| Luo | J. | 110 | Michaux | P. | 18 |
| Lutz | F. | 201 | Michel | G. | 13 |
| Ma | S. | IV | Michelena | G. | 191 |
| Mack | K. | 109 | Mietheke | N. | 195 |
| Madhusudhan | K. | 110 | Mietling | R. | 126 |
| Mansfield | J. W. | 87 | Min | K-H. | 75 |
| Manzanera | M. E. | XVII | Moebus | E. | 82, 119 |
| Maraver | A. | 63, 64 | Molm | W. W. | 113 |
| Marconi | A. M. | 85, 111, 134 | Mol | C. D. | 72 |
| Marqués | S. | XVII | Molin | S. | VIII, 29, 116, 151, 209 |
| Mars | A. E. | IX, 112 | Molina | L. | 151, 209 |
| Martin | V. | 113 | Molle | G. | XLII, 54 |
| Martin | N. | 22 | Moller | S. | VIII, 29 |
| Martin | P. R. | XXI | Montebove | F. | 160 |
| Martinez | A. | 114 | Moore | E. R. B. | 21 |
| Martinez | J. L. | 3, 4 | Morgan | A. | 169 |
| Martino | M. | XII | Morgan | M. | 207 |
| Martinotti | M. G. | 115 | Morgan | A. J. | 39, 73 |
| Martins-Dos-Santos | V. A. D. | 62 | Mosqueda-Cano | G. | 62 |
| Maser | E. | 82, 119 | Mossialos | D. | 57, 120 |
| Mason | J. R. | 12 | Moxon | E. R. | 92 |
| Mathee | K. | IV, 116 | Mrachko | G. T. | 34 |
| Mathys | R. | XXXVII | Mulders | I. | XX, 38 |
| Matthijs | S. | XXIV | Müller | C. | 104, 121, 122 |
| Mattick | J. | XXI | Müller | P. | 158 |

| | | | | | |
|-----------------|-----------|--------------------------------------|------------------|-----------|-------------------|
| Muñiz | L. M. | 63, 64 | Olson | P.E. | 117 |
| Murillo | J. | 27, 87, 167, 168 | Olvera | C. | 172 |
| Myyashita | K. | 132 | On | H.Y. | 75 |
| Nacamuli | C. | 33 | Opes | F. | 201 |
| Nakahara | T. | 2, 123, 128 | Oppenheim | A.B. | 15 |
| Nakajima-Kambe | T. | 2, 123, 128 | Orange | N. | XLII, 54 |
| Nakayama | T. | 185 | Ornston | N. | XIII |
| Nakazawa | T. | XXX | Oropeza | A. | 142 |
| Neish | B. | 124 | Orsi | N. | 106 |
| Nezezon | J. | 36 | Ortiz-Barredo | A. | 167 |
| Ng | L. C. | 135 | Ostrovsky | P. | 152 |
| Nichols | N.N. | XXXIX | Overhage | J. | 136 |
| Nicolo` | M. | 70 | Pain | A. | 171 |
| Niehaus | K. | 118 | Pan | J-G | 91 |
| Nielsen | M. | 126 | Pan-Hou | H. | 85 |
| Nikaïdo | H. | XL V | Pang | B.C.M. | 137, 184 |
| Nishi | A. | 125 | Panke | S. | 138 |
| Nishijyou | T. | 127 | Parales | J. | XXXVI |
| Nishino | T. | 66 | Park | S. | 127, 139 |
| Nishiyama | K. | 164, 193 | Passador | L. | XXVII, 36 |
| Nomura | N. | 2, 66, 123, 128 | Pavlu | L. | 140, 149 |
| Norman | R. | XXXV , 129 | Pearl | L. | XXXV , 129 |
| Nowak-Thompson | B. | 6, 130 | Pearson | J.P. | XXVII, 141, 186 |
| Nunn | D. | 114, 152 | Pechère | J.C. | 97 |
| Nurk | A. | 143, 144 | Pedrota | V. | 138 |
| O'Gara | F. | XXIII , XLII , 23, 103 | Pelizzoni | F. | 41 |
| O'Hara | B. | XXXV , 129 | Peñaloza-Vazquez | A. | 142 |
| O'Leary | N. | 134 | Perera | J. | 188 |
| O'Neill | E. | 135 | Pérez-Martín | J. | XVI |
| O'Sullivan | O. | 23 | Pernice | A. | 69, 70 |
| Ochs | M. | XLI , 131 | Perucha | E. | 63, 64 |
| Ogawa | N. | 132 | Pesci | E. | XXVII, 141, 186 |
| Ohman | D. | IV , 94, 116 | Peters | M. | 143, 144 |
| Ohnishi | K. | XXXIII | Petruschka | L. | 121 |
| Okamoto | K. | 66 | Petruzzeli | R. | 48 |
| Okuta | A. | XXXIII | Phibbs | P. V. | 71, 72, 148 |
| Olsen | R. | 133 | Phoelis | C. | XX, 38 |

| | | | | | |
|------------------|-------|--------------------------------|--------------|----------|--------------|
| Pieper | D. | 21, 88, 96, 187, 194, 208, 214 | Ronchel | M.-C. | 209 |
| Pinti | M. | 9 | Rotilio | D. | 48 |
| Ponente | A. S. | 160 | Rudolph | K. W. E. | 78, 107, 158 |
| Pongsunk | S. | 43, 145 | Russel | S. | 159 |
| Poole | K. | XLIV, 175, 176, 199 | Ruigers | M. | XV |
| Potrawfke | T. | 146 | Ruzzi | M. | 111, 160 |
| Priefert | H. | 136 | Sabarth | N. | 89 |
| Prieto | M.A. | 147 | Saddler | J. N. | 113 |
| Prò | A. | 85 | Safrin | M. | 94 |
| Proctor | W. D. | 148 | Sam | L. | 161 |
| Prouza | M. | 149 | Santos | P. | 84 |
| Provoost | A. | XI | Sarand | I. | 162 |
| Pühler | A. | 118 | Samignet | A. | 37, 163, 196 |
| Pujana Uriarte | I. | 150 | Sato | M. | 164, 193 |
| Puntus | I. F. | 52 | Sato | Y. | 165 |
| Puyet | A. | 31 | Schell | M. | XXII |
| Qingsheng | Q. | XL, 101 | Schirmer | A. | 166 |
| Raajmakers | J. W. | 185 | Schmid | R. | 119 |
| Rabenhorst | J. | 136 | Schmid | A. | XXXVII, 138 |
| Rainey | P. | II, 58, 92, 124 | Schmidt | T. | XXVIII |
| Ramos | C. | 151, 209 | Schmidt | K. D. | XXVIII, 173 |
| Ramos | J. L. | XVII, 62, 151, 209, 212, 213 | Schneidinger | B. | 105, 197 |
| Ratliff | M. | 152 | Schoofs | G. | 32 |
| Ravatin | R. | 153 | Schweizer | H. P. | XLIII |
| Regeard | C. | 211 | Segura | A. | XIII |
| Rehm | B. | XL, 101, 154 | Selifonova | O. V. | 42 |
| Reifenrath | C. | 30 | Sello | G. | 41 |
| Reineke | W. | IX, 112 | Sesma | A. | 27, 167, 168 |
| Ren | Q. | 155 | Seveno | N. | 169 |
| Riegert | U. | 156 | Shepherd | J. | 170 |
| Rigal | A. | 212 | Shigeno | T. | 128 |
| Rodriguez-Hervas | J-J | 62, 212 | Shingler | V. | V, 135, 177 |
| Rojó | F. | 204 | Sierralta | W. | 195 |
| Romagnolo | O. | 70 | Simons | M. | XX |
| Romantschuk | M. | 162 | Sinha | H. | 171 |
| Römling | U | XXVIII | Skovasch | D. | 82 |
| Rompf | A. | 157 | Smith | J. | 171 |

Enzyme Superfamilies: Oxygenase Electron Transport Flavoproteins

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Microbes have been recognized for more than a century as primary, essential agents in the ecology of carbon flux and mineralization in nature. Oxygenases, abundantly documented in microbes and broadly distributed in ecosystems, are equally important to biosyntheses and detoxication. The terminal oxygenase proteins are coupled to a variety of reductases with homology to the essential enzymes of respiration, photosynthesis and pathways of anaerobic equivalence.

Molecular analysis of protein and DNA sequences using computational programs and improved data bases enhances the understanding of structure and phylogeny. We selected 55 of the better characterized electron transport flavoproteins to examine common reductive features of a variety of oxygenases and anaplerotic enzymes. The oxygenase reactive centers accept two reducing equivalents to form reactive intermediates that bind sequentially with carbon and dioxygen substrates. Many, perhaps most, contain a metal, often iron in heme, dissociable ferrous iron or the $\text{Fe}_2(\text{mO})_2$ as in methane monooxygenase.

The organization of the oxygenase reductase components extends beyond the flavine and pyridine nucleotide loci; some contain a covalent N-or C-terminal, $\text{Fe}_2\text{S}_2\text{Cys}_4$ redoxin, others, a N-terminal covalent region with FMN. The latter are found in hepatic microsomes. The redoxins may occur also as small, separate plant/chloroplast type proteins. For example, as found in prokaryotic non-heme oxygenases and microbial-mitochondrial type II P450 heme-thiolate monooxygenase. A second higher potential redoxin of Rieske type, $\text{Fe}_2\text{S}_2\text{His}_2$, may also appear as a separate electron transport protein, in oxygenase center complexes, or in both, and most particularly in dissociable ferrousiron oxygenases. Broadening the analysis to mono- and dioxygenases and anaplerotic flavoproteins revealed two patterns, tentatively termed Red1 and Red2. These reflect distinct homology patterns of conserved domains, crystal structure, and apparent phylogeny. A deeper analysis of structures, genetic exchange patterns, and organization in oxygenase center identities appears warranted and useful.

Gene regulation in the biocontrol strain *Pseudomonas fluorescens* F113 in response to environmental signals

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The biocontrol strain *Pseudomonas* F113 produces the potent antifungal metabolite 2,4-diacetylphloroglucinol (Phl). The nature and putative function of the biosynthetic and regulatory *loci* involved in Phl synthesis have been determined to the genetic level. This research has identified a complex regulatory cascade which controls Phl biosynthesis. The Phl regulatory cascade includes a two component sensor response-regulator, quorum sensing, alternate sigma factors, a negative regulator and an additional novel regulatory gene. In addition, the synthesis and regulation of *P. fluorescens* antifungal metabolites are influenced by numerous environmental factors such as temperature, carbon source and iron availability. However, within the plant rhizosphere there are further more complex interactions occurring, including those between the indigenous colonising microflora and also between the microflora and the plant root. The BIOMERIT laboratory has evaluated many of these interactions and demonstrated that gene regulation in *P. fluorescens* is significantly influenced by bacterial-bacterial, plant bacterial and fungal-bacterial signalling.

In our most extensively evaluated system, F113 genes which exhibited differential expression in response to fungal signals (using *Pythium ultimum* as a model strain) have been isolated. The signal responsive genes have been characterised in a series of detailed phenotypic and genotypic studies. Results from these investigations have revealed that functions involved in nitrogen metabolism and cell growth rate are significantly repressed. The nature of the *Pythium* signal (s), the plant-bacterial and bacterial-bacterial signals are currently being investigated. Furthermore the role of these signal metabolites in the production and regulation of antifungal agents in *P. fluorescens* is also being evaluated.

Population biology and evolution of *Pseudomonas* bacterial lung colonization in cystic fibrosis

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The involvement of the mucoid colonial morphotype of *Pseudomonas aeruginosa* in the lung colonization/infection of cystic fibrosis (CF) patients is a well recognized fact. Nevertheless, most CF patients harbour several colony morphotypes. Morphotype diversity in other patients remains rare, and is only occasionally found in CF-like pathology, as chronic bronchitis with bronchiectasis. Interestingly, in our series, the number of different colonial morphotypes per sputum sample increases with the age of the CF patient, and patients with higher number of morphotypes have low scores of lung function and general status (FVC, FEV1, Tiffenau, Schwachman). Auxotrophy in *P. aeruginosa* is also rarely found outside CF isolates, nevertheless, we found in our series that nearly half of the patients harboured auxotrophic (mostly methionine) auxotrophs. Similarly as in the case of colonial morphotypes, the patients with auxotrophs tended to correspond to the more advanced cases, with deeper lung deterioration. Cluster analysis of phagetypes of series of isolates from every same clinical case suggests that every patient is colonized during prolonged period of time with a reduced number of clones. Therefore, the increase in diversity apparently occur within particular clones. The origin of diversity may be related with generation of variation under bacterial stress/stationary phase (mutator genes-contingency loci?). In advanced CF patients, many of these variants seems to find the opportunity to be fixed. Probably the progression of the lung deterioration produces habitat fragmentation (compartmentalization). Small and diverse compartments may increase the rate of fixation of particular genotypes. That supports the clinical use of the number of morphotypes as a marker of lung deterioration. A mixture of colonial morphotypes in broth under agitation (homogeneous environment) leads to the dominance of a single colonial morphotype, but using a more structured-compartmentalized environment (cooked meat balls), a higher diversity of morphotypes is recovered. Some of these types colonize preferentially particular areas of stationary broth spatially heterogeneous environment (Railey and Travisano model). The distribution of *Pseudomonas* populations of small size in multiple-small compartments emerging during lung deterioration may produce a random drift (like sampling-error) effect that also contribute to the fixation of particular variant phenotypes. Finally, some types may create a self-selective environment; in minimal medium Davis with alginate 0.05 % the mucoid phenotype do not revert to non-mucoid, and competition with other variants goes in favour of the former. The evolution of *Pseudomonas* populations in CF patients may reflect the progression of changes in the lung environment.

A diffusible factor produced by the phytopathogen *Pythium ultimum* represses expression of genes required for key metabolic functions in *Pseudomonas fluorescens* F113

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There is increasing evidence that communication between members of the same species as well as members of other species is important for the survival of microorganisms in diverse ecological niches. In our group this observation has recently been extended to interactions between bacterium and a fungus indigenous to the sugarbeet rhizosphere. Using Tn5::lacZ mutagenesis five gene clusters were identified in the biocontrol strain *P. fluorescens* F113 which were repressed in the presence of the phytopathogenic fungus *Pythium ultimum*...

The ability of the reporter mutants to colonise the sugarbeet seed/root system and to survive in the sugarbeet rhizosphere was impaired for each of the mutants. This was not due to a defect in their biocontrol abilities. Phenotypic characterisation of the reporter mutants revealed that all had similar growth requirements as the wild-type with the exception of SF10. The impaired colonisation ability of this mutant was due to a defect in glutamate synthase (GOGAT), an enzyme important for growth under ammonia deficient conditions. The transposons in two of the reporter mutants mapped within ribosomal RNA operons. The insertion occurred within the 23s rDNA gene in SF3 and within the 16s rDNA gene in SF5. Differential regulation of hte promoters in SF3 and SF5 indicate that different rRNA operons are affected. Preliminary characterisation of these mutants suggest that their reduced rhizosphere competence is not due to impaired growth but rather impaired viability. This current data indicate the *P. ultimum* derived metabolites can repress key metabolic functions involved in protein synthesis. This could be a potential mechanism by which *P. ultimum* influences the establishment of the biocontrol strain *P. fluorescens* F113 in the sugarbeet rhizosphere.

5th September, Poster 59bis

A general system for isolation of large and unlabeled deletion mutations in *Pseudomonas* chromosomes

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A class II transposon, Tn1721, encodes a site-specific resolution system, in which the resolvase (TnpR) catalyzes intramolecular recombination between the two directly oriented copies of the resolution (*res*) site, leading to precise excision of the intervening DNA region. This property was exploited to develop a general strategy to isolate the large chromosomal deletion mutations with defined endpoints. Such a mutation could be obtained by a two-step manipulation of the target chromosome using the two cloned chromosomal fragments with small sizes. In the former step, a *res-Ω* cassette inserted in a cloned chromosomal fragment was transferred to the target chromosome by allelic exchange. In the latter step, a ColEI-type plasmid carrying another chromosomal fragment and a *res-tnpR-tet* cassette was integrated into the target chromosome through single crossover-mediated homologous recombination. The plasmid integrant carrying the two copies of the *res* site in the same orientation generated very efficiently the segregants in which the chromosomal fragment flanked by the two *res* sites was precisely excised through the TnpR-mediated site-specific resolution. The deleted chromosomal fragment in such segregants was replaced by the *tet* or *Ω* gene. Suitable combination of the *res-Ω* and *res-tnpR-tet* cassettes along with the appropriate selection conditions further allowed successful construction of the deletion mutants that lacked both the *tet* and *Ω* genes. The deletion system was applied to isolate large deletion mutations in the *pvd* region of *P. aeruginosa* PAO that carries most of the pyoverdine biosynthetic genes. Successful isolation of a 106-kb deletion mutation indicated that the *pvd* region did not carry any essential genes. Another successful example of the system is presented by Gotoh *et al.* in this Symposium.

5th September, Poster 33

Regulation of elastase production in *Pseudomonas aeruginosa* :Evidence for a role for PvdS in the regulation of *lasA* and *lasB* expression

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Previous studies have shown that the genes required for elastolytic activity by *P. aeruginosa*, *lasA* and *lasB*, are under complex regulatory control involving the transcriptional activator LasR and *P. aeruginosa* autoinducer (PAI) encoded by the *lasI* gene is responsible (1). PAI (N-(3-oxododecanoyl) homoserine lactone) is a member of a family of N-acylhomoserine lactone compounds which are involved in gene activation in response to cell density. It has also been suggested that the ECF sigma factor, PvdS, required for pyoverdinin production may also been involved in the regulation of the virulence factors in *P. aeruginosa*. The *pvdS*, required for pyoverdinin production may also be involved in the regulation of virulence factors in *P. aeruginosa*. The *pvdS* gene showed >80% identity, at the DNA level, with the *Pseudomonas fluorescens* sigma factor gene, *pbrA*, which was previously found to coordinately regulate siderophore and protease production in *P. fluorescens* sigma factor gene, *pbrA*, which was previously found to coordinately regulate siderophore and protease production in *P. fluorescens* M114 (2). Subsequently, PvdS was found to be required for exotoxin A production probably via the *regAB* promoters (3) and enzyme assays indicated reduced levels of extracellular metabolite production, including proteases and elastase, by Δ pvdS *P. aeruginosa* mutant (4).

In order to investigate if *PvdS* is involved in the transcriptional regulation of *lasB* expression, the kinetics of the expression of *lasA* and *lasB* reporter fusions (*lasA-lacZ-lasB-lacZ*) were assessed in wildtype and *pvdS* deleted *P. aeruginosa* backgrounds. The expression of *lasB* in the *PvdS* background was reduced to, on average, 25% of wildtype levels from early log through stationary phase of growth (4–18 hours). However, *lasA* expression in the *PvdS* background was not altered during early to mid during late log phase of growth but was reduced to approximately 50% of wildtype levels during late log through stationary phase (12–18). Wildtype levels of expression were restored when the active *pvdS* gene was introduced in *trans*. Interestingly, introduction of the active *pbrA* gene did not restore *lasA* or *lasB* expression levels.

This study confirms that the ECF sigma factor, PvdS, is required for full expression of *lasA*. The expression levels of *lasA* are more dependent on PvdS than *lasB* and *lasB* genes *P. aeruginosa*. The expression of *lasA* appears to occur only during late log and stationary phase. It is also indicated that PvdS and PbrA may differ in their functionality.

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- Fifth International Symposium on *Pseudomonas*: Biotechnology and Molecular Biology (1995) Abstract: F-10

Regulation of the biocontrol metabolite 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* F113

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The biocontrol ability of *P. fluorescens* F113 is conferred through the production of the potent antifungal metabolite 2,4-diacylphloroglucinol (Phl). Detailed analysis of a Phl biosynthetic locus previously identified and isolated from the F113 genome was completed. Characterisation of the biosynthetic locus by sequence analysis identified at least four putative open reading frames involved in the biosynthesis of Phl and a divergently transcribed locus-linked negative regulator specific for the repression of Phl biosynthesis. This putative repressor shows similarity to other well characterised repressors including TetR.

summary to conclude that constitutive repressors influence the growth of *E. coli*. Our recent studies have largely focused on the role of this putative repressor on Phl biosynthesis. Addition of multicopies of the putative repressor to the F113 wildtype significantly reduced Phl production. This effect was shown to be mediated at a transcriptional level using a Phl biosynthetic gene:*lacZ* fusion. Expression of Phl in F113 is growth phase dependent. Inactivation of the repressor gene (*phlR*) in F113, resulted in constitutive Phl production. A truncated clone, from which the putative repressor was removed exhibited constitutive Phl production in the wildtype and Phl biosynthetic mutant backgrounds. Removal of the Phl negative regulator also decouples Phl synthesis from *ApdA/GacA* two component regulator control.

Phil production was conferred to all heterologous *Pseudomonadaceae* backgrounds assessed upon introduction of the truncated clone. This offers the potential for the construction of novel GMO's expressing multiple biocontrol traits.

Studies on mechanisms of induced resistance by bacterial lipopolysaccharides (LPS) in *planta*

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Several authors reported that treatment of leaves with bacterial lipopolysaccharides (LPS) induced resistance against subsequent "challenging" inoculation with intact bacteria (1,2). However, the underlying mechanisms have never been completely unravelled. It is yet unknown which component of the LPS (core-region, O-chain or lipid A) is responsible for this effect. Our studies aimed to elucidate the interactions between surface molecules of plants and bacteria in more detail by investigating the effects of LPS-subunits and analysing the chemical structures of the components involved.

Lipopolysaccharides (LPS) were extracted from the following *Pseudomonas syringae* pathovars: tomato race 1 (GSPB Nr. 1778), tomato race 0 (GSPB Nr. 1776), and glycine race 9 (GSPB Nr. 1986) by the phenol/chloroform/petroleumbenzene-method (4). The chemical analysis amounts of KDO, PO₄³⁻, GlcNAc and rhamnose. Chemical structure-analysis identified the O-specific chain, the core region and lipid A. Lipid A was composed of the typical fatty acids of *Pseudomonas* such as 3-OH-C10:0, C12:0, 3-OH-C12:0 and 2-OH-C12:0. The typical structure of the O-chain corresponded to the one reported by (3), typical for *Pseudomonas syringae* pv. *glycinea*. Determination of the core-region has not been completely finished, but seems to show strong similarities to *P. aeruginosa* and *P. fluorescens*.

Induced resistance was studied in tomato and tobacco leaves. We investigated the role of whole LPS as well as its subunits O-chain and core region on induced resistance in compatible and incompatible systems. Resistance was induced by 50 µg LPS/ml in the incompatible system (lps OF *P. s. pv. glycinea* against *P. s. pv. tomato* race 1 in tomato cv. "Lycanorma"). Neither the O-specific chain nor the core region alone induced resistance. When similar experiments were performed by pretreatment with *P. s. pv. tomato* LPS followed by inoculation with *P. s. pv. tomato* (compatible system) resistance was not induced. Tobacco leaves were pretreated with 50-1000 µg LPS/ml of *P. s. pv. glycinea* followed by inoculation with 108 cfu/ml of *P. s. pv. glycinea* 48 h later. The pretreatment delayed appearance of the HR from 20 to 60 hrs after bacterial inoculation. The O-chain or the core-region did not cause this effect. Studies of a rough mutant *P. s. pv. tomato*-LPS in tobacco showed effects when tobacco leaves were pretreated with only 5 µg/ml LPS. Analysis of this rough mutant LPS showed small quantities of O-chain, so we concluded a possible involvement of lipid A in induced resistance phenomena. We tested lipid A of *P. aeruginosa*, *E. coli* and *P. s. pv. tomato*. In lipid A-pretreated tobacco leaves HR was delayed for 2 days after challenge inoculation with *P. s. pv. tomato*. Further experiments have to show why the plants can differentiate between compatible and incompatible LPS, and whether the complete LPS-molecule is necessary for resistance induction or whether lipid A alone can cause this effect.

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6th September, Poster "a"

Of *Pseudomonas* and Nitrosamines

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Microbial nitrate reductase activity has been widely studied for several reasons. Besides theoretical interest in nitrate reduction the main interest lies especially in the dissimilative nitrate reductase activity of bacteria which plays a significant role in soil and water systems. In the field of food industry the nitrate-reducing bacteria represent one of the most undesirable forms of food contamination, as the enzymatic reduction of nitrate to nitrite and A-oxides facilitates further formation of the carcinogenic A-nitrosamines, known under the abbreviation of ATNC (Apparent Total A-Nitrosocompounds). Three major types of microbial reductases can be distinguished, according to the type of nitrate utilisation they are part of: to their localisation in the cell and their enzymatic function and properties: they are the assimilatory nitrate reductase (NAS), the dissimilatory (respiratory) nitrate reductase (NAR) and the periplasmic nitrate reductase (NAP). *Pseudomonas pseudocaligenes* originally isolated from hopped wort, was chosen for the isolation and purification of nitrate reductase due to high production of this enzyme. Nitrate reductase from this strain was characterised as assimilatory nitrate reductase (EC 1.6.6.1), and was localised in cytosol. At eleven food samples the enumeration of viable microorganisms was estimated and the ratio of bacterial strains exhibiting nitrate reductase activity was determined. In all food samples the presence of ATNC was evaluated, and the these findings were compared with the number of nitrate reductase positive strains.

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6th September, Poster "b"

Holistic bottom up and top down approaches to elucidate genome differences
between two clonal variants of *Pseudomonas aeruginosa*

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The comparative analysis of the genome organization of clonal variants of *P. aeruginosa* unravels the genetic diversity at the level of minimal phylogenetic divergence. The two *P. aeruginosa* strains selected for the study occupy distinct ecological habitats. That is C, a cystic fibrosis (CF) patient isolate, and SG17M, an environmental isolate, both belonging to the same predominant *P. aeruginosa* clone.

Strain-specific DNA sequence tags were isolated by subtractive genomic hybridization, whereby both strains were used as target and tester. After three cycles of subtraction, subsequent PCR amplification and cloning in pBluescript vector, a total of 35 specific subtraction clones (200 to 550 bp in size) was obtained as confirmed by Southern hybridization on *Pst*I-digested genomic DNA.

Physical and genetic maps were constructed by two-dimensional PFGE methods for the 6.5-Mb circular chromosomes of *P. aeruginosa* C [Schmidt *et al.*, 1996] and SG17M. Twenty-five genes were placed on to the *Spe*I-, *Pac*I-, and *Ceu*I-macrorestriction maps by Southern hybridization. Comparison of the chromosomal maps revealed conservation of gene order, but insertions and deletions of large blocks of genetic material gave rise to a mosaic-like genome structure. A total of 340 kb DNA was mutually absent from each chromosome and present in the other genome.

All 35 subtraction clones hybridized to genomic regions that are different in the physical maps. Some clones gave additional signals on *Spe*I fragments identical in both strains. Subsequently, a selection of 6 SG17M-specific and 11 C-specific subtraction clones was sequenced. The GC-content of 5 inserts was below 50%, of 10 inserts between 50 and 60%, and of the remaining two inserts 62% and 64%, respectively. All values are below the mean GC-content of 67% in the *P. aeruginosa* genome. The comparison of the nucleic acid sequences and the protein sequences translated from all possible open reading frames (ORFs) of 15 clones with the current databases revealed no significant homology. Only one clone shares high homology to an uncharacterized ORF of *P. putida* and another clone is identical to a part of an insertion element. Northern hybridization was used to determine if the subtraction clones encoding ORFs are expressed under various culture conditions. The on-going characterization of cosmid clone banks of the corresponding genomic regions will enable further analysis of interesting genomic differences and of underlying genetic events.

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6th September, Poster "c"

Pseudomonas aeruginosa infection in cystic fibrosis:
animal model of the transgenic *cfr^{neu}* mouse

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Objectives: *P. aeruginosa* infection of the respiratory tract plays a pivotal role in morbidity and mortality of Cystic Fibrosis (CF) patients. A standardized animal model of the *P. aeruginosa* infection will be helpful to determine the *in vivo* virulence determinants increased by the bacteria in the CF lung habitat and to develop new therapeutic guidelines for the treatment and prevention of the chronic *Pseudomonas* infection in CF.

Methods: Transgenic *cfr^{neu}* mice, with residual mRNA expression of approximately 10-15% in lung tissues, were consecutively infected with *Haemophilus influenzae*, as early colonizer, and *P. aeruginosa* by aerosol nebulization. Bacterial colony counts, differential cell counts in bronchoalveolar lavage and peripheral blood smear as well as immunocytological staining for inflammatory cells in lung tissue were used to characterize acute and repeated infection.

Results: Repeated aerosolization of *P. aeruginosa* for 28 consecutive days induces an inflammatory response in lung tissues. An early efflux of polymorphonuclear neutrophils (PMN) from blood to the alveolar lumen can be observed within 8 hours. PMN were the predominant inflammatory cells in lung tissue as well as in the alveolar lumen. Inflammation resolves by day 5 post infection. Bacteria counts immediately after nebulization were 10¹⁴ per lung, decreasing to 10³ at day 5 after cessation of aerosolization. At the latest time point, 21 days post infection, 10³ germs were lavageable from the lungs. The *Pseudomonas* neither switch to a mucoid phenotype nor do they alter their genotype. Inflammatory response and kinetics of bacterial counts were the same in transgenic as in control mice.

Discussion: With the chosen experimental design, we were not able to induce a persistent colonization of the respiratory tract of mice. Even gene targeting in the murine *cfr* did not render the animals susceptible to a chronic *P. aeruginosa* infection. We conclude that genetic factors other than *cfr* and/or preceding exposure to other infections and/or noxious agents may be necessary to establish a chronic infection in the transgenic *cfr^{neu}* mice.

6th September, Poster "d"

MOLECULAR STRUCTURE AND REGULATION OF THE POLYESTER DEGRADING SYSTEM OF *PSEUDOMONAS LEMOIGNEI*

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Pseudomonas lemoignei was isolated 1965 as one of the first poly(3-hydroxybutyrate) (PHB) -degrading bacteria and was named in honour to Maurice Lemoigne who had discovered PHB as a constituent of bacteria in 1925. *P. lemoignei* belongs to the beta subclass of proteobacteria and is related to the *Burkholderia-Ralstonia* rRNA sublineage. The metabolic capabilities of *P. lemoignei* are restricted to the utilization of a few organic acids and polyesters such as PHB, poly(3-hydroxyvalerate) (PHV) and related polyhydroxy-alkanoates (PHA) [1].

The degradation of PHA depends on the formation of extracellular enzymes (PHA depolymerases), which hydrolyze the polyesters to water-soluble products, and has been reviewed recently [2]. Six different polyester depolymerase genes (*phaZ1* - *phaZ6*) have been cloned in *P. lemoignei* and code for six PHA depolymerases which slightly differ in their substrate specificities. PHA depolymerases resemble serine-hydrolases and are similar to lipases, esterases or serine-proteases with respect to their catalytically active amino acids (catalytic triad). In addition to the catalytic domain the depolymerases consist of a C-terminal substrate-binding domain which enables them to bind specifically to the water-insoluble polyester.

The synthesis of PHA depolymerases is highly regulated in most PHA-degrading bacteria, and PHA depolymerase expression is repressed in the presence of suitable soluble carbon sources. In contrast, PHB depolymerase formation in *P. lemoignei* is stimulated during growth on succinate [3]. Growth studies on succinate at various pH values and transport experiments using ¹⁴C-labelled succinate revealed that growth rates and uptake of succinate are maximal between pH 5.6 and 6.5 and strongly reduced above pH 7. pH-shift experiments, inhibition studies using KCN, NaN₃, maleimide, tetrachlorosalicylanilide (TCS) and osmotic shock experiments gave evidence for the absence of an H⁺/succinate symporter but for the presence of a periplasmatic located pH-dependent succinate-specific ABC transporter. We conclude that expression of PHB depolymerases in *P. lemoignei* during growth on succinate is due to unsufficient uptake of succinate as soon as the pH becomes higher than 7 resulting in carbon starvation and derepression of PHB depolymerase synthesis.

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7th and 8th September, Poster "b"

THE DNA BINDING REGION OF THE XylS PROTEIN AND ITS TARGETS AT THE Pm PROMOTER

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Pseudomonas putida 2440 bearing TOL plasmid pWW0 is able to grow on 3-methyl benzoate (3MBz) as the sole carbon source using the *meta* cleavage pathway. This pathway is encoded by the *meta*-cleavage operon expressed under the control of the Pm promoter. Activation of Pm is dependent on effector activated XylS protein.

It has been proposed that the XylS binding site may be represented by the motif T(C or A)CAN₄TGCA, which appears twice in the promoter sequence, and consists of two submotifs (TNCA) separated by 4 bp that are simultaneously direct and inverted repeats. Point and multiple mutations at the proposed binding region in Pm were generated by overlap-extension PCR mutagenesis. The mutant promoters obtained were fused to 'lacZ' in pMD1405, and the expression from these was measured as β -galactosidase activity in cells growing in the absence and in the presence of 3MBz. The results obtained corroborated the organization of XylS binding sites in the Pm promoter and suggested that the proximal site (-46/-57), adjacent to the RNA polymerase binding site, constitutes the minimum sequence required for transcription stimulation. The second XylS binding motif further upstream (-67/-78) may be responsible for increasing DNA occupancy, thus enhancing transcription from Pm.

The XylS protein belongs to the so-called XylS/AraC family of transcriptional regulators. In XylS, six α -helices (α -H) were predicted; four of them: α -H 2 and 3 and α -H 5 and 6 are organized as two α -helix-turn- α -helix (HTH) motifs, that are conserved among the members of the family. In order to determine the role of α -H 5 and 6, we have used site directed mutagenesis to generate a series of single mutants in residues that form part of these helices. The ability of these mutants to activate transcription from wild type and the above described mutant Pm promoters has been analyzed. Evidences are shown suggesting that this region of XylS is essential to promote transcription.

7th and 8th September, Poster "a"

Genetic systems for organosulfur utilization by *Pseudomonas aeruginosa*

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Less than 5% of the sulfur present in soil is in the form of inorganic sulfate, and pseudomonads are therefore required to assimilate organosulfonates and organosulfate esters to obtain sulfur for growth. We studied the response of *Pseudomonas aeruginosa* to sulfate-starvation conditions, and found that several proteins were upregulated, including the sulfate-binding protein, a putative thiol-specific antioxidant, and systems for utilization of organosulfur compounds. The *slf/ABC* operon was identified from the protein sequence of one of these sulfate-regulated proteins, and appears to play a role in sulfonate utilization. *slfA* and *slfB* showed little homology to published sequences, but probably encode a monooxygenase system (1). They are closely related to *E. coli* desulfonation genes we are also studying. *SlfC* is 40% identical to a sulfide/sulfoxide oxygenase active in dibenzothiophene desulfurization. Using a *slfB::xylE* fusion we found 100-fold upregulation of gene expression in the absence of sulfate, with control at a transcriptional level. No sulfur-utilization phenotype could be found for insertion mutants in *slfB*, suggesting that there may be two desulfonation systems present in the cell, as previously shown for *E. coli* (2).

Mutants in the *slf* locus showed reduced levels of arylsulfatase activity, suggesting a regulatory inter-relationship between these two sets of genes. The arylsulfatase gene (*atsA*) is encoded as part of a four-gene cluster (*atsRBCA*), and has been shown to be strongly repressed during growth with sulfate (3). *atsR* is encoded divergently to *atsBCA*, and may have substrate-binding or regulatory function, whereas the *atsBC* genes show homology to genes encoding ABC-type transport systems.

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